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<b>(54) Title:</b> CHIMERIC PLANT GENES BASED ON UPSTREAM REGULATORY ELEMENTS OF HELIANTHININ		
<b>(57) Abstract</b> <p>Helianthinin is an 11S seed storage protein of sunflower embryos. The present invention is directed to the 5' regulatory regions of helianthinin genes. More particularly, the present invention is directed to specific cis-regulatory elements of this regulatory region which direct tissue-specific, temporally-regulated, or abscisic acid-responsive gene expression. The present invention provides chimeric genes comprising the cis-regulatory elements linked to a coding sequence from a heterologous gene to control expression of these genes. The chimeric genes provided by the instant invention are useful in conferring herbicide resistance and improved seed lipid quality to transgenic plants.</p>		

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CHIMERIC PLANT GENES BASED ON UPSTREAM  
REGULATORY ELEMENTS OF HELIANTHININ

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Helianthinin is an 11S seed storage protein of sunflower embryos. The present invention is directed to the 5' regulatory regions of helianthinin genes. More particularly, the present invention is directed to specific cis-regulatory elements of this regulatory region which direct tissue-specific, temporally-regulated, or abscisic acid-responsive gene expression. The present invention provides chimeric genes comprising the cis-regulatory elements linked to a coding sequence from a heterologous gene to control expression of these genes. The chimeric genes provided by the instant invention are useful in conferring herbicide resistance and improved seed lipid quality to transgenic plants.

Seed development, unique to higher plants, involves embryo development as well as physiological adaptation processes that occur within the seed to ensure the survival of the developing seedling upon germination. After fertilization, there is rapid growth and differentiation of the embryo and endosperm, after which nutritive reserves accumulate during the maturation stage of seed development. These reserves are stored during a period of developmental arrest for later use by the developing seedling. This period of arrest occurs prior to the desiccation phase of seed development.

Several classes of seed proteins, including storage proteins, lectins, and trypsin inhibitors, accumulate during embryogenesis. The main function of seed storage proteins is to accumulate during embryogenesis and to store carbon and nitrogen reserves for the developing seedling upon germination. These proteins, as well as many of the genes encoding them, have been studied extensively (for review see Shotwell et al. (1989) in The Biochemistry of Plants, 15, Academic Press, NY, 297).

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1 Genes encoding seed storage proteins are highly  
regulated and differentially expressed during seed development.  
Expression is temporally regulated with mRNA accumulating  
rapidly during the maturation phase of embryogenesis. This  
5 expression is also tissue-specific, occurring primarily in the  
cotyledons or endosperm of the developing seeds. The resulting  
storage proteins are processed and targeted to protein bodies,  
in which the storage proteins remain during desiccation and  
dormancy of the embryo. Upon germination, the seedling uses  
10 these storage proteins as a source of carbon and nitrogen  
(Higgins (1984) Ann. Rev. Plant Physiol. 35, 191).

Seed proteins, including storage proteins, lectins and  
trypsin inhibitors, are encoded by nonhomologous multigene  
families that are not amplified or structurally altered during  
15 development (for review see Goldberg et al. (1989) Cell 56,  
149). These genes are temporally and spatially regulated but  
not necessarily linked. Although post-transcriptional  
mechanisms act to control the accumulation of some of these  
proteins, regulation occurs primarily at the transcriptional  
20 level. Accordingly, seed protein genes provide an excellent  
system to provide genetic regulatory elements, especially those  
elements which confer tissue specificity, temporal regulation,  
and responsiveness to environmental and chemical cues.

Observations of temporal and spatial regulation of  
25 seed protein genes has suggested that seed protein genes are  
regulated in part by common cellular factors known as trans-  
acting factors. However, since quantitative and qualitative  
differences exist in the expression patterns of individual seed  
protein genes, more specific factors must also exist to provide  
30 a means for differential expression patterns between these  
groups of seed proteins. Patterns of differential expression  
have been observed between the rapeseed major seed storage  
proteins, cruciferin and napin (Crouch et al. (1981) Planta 153,

1 64; Finkelstein et al. (1985) Plant Physiol. 78, 630), and among  
individual members of the soybean Kunitz trypsin inhibitor gene  
family (Jofuku et al. (1989) Plant Cell 1, 1079). A comparison  
of the soybean major seed storage protein genes showed a  
5 difference in timing and cell-type specificity of the expression  
of  $\beta$ -conglycinin (7S) and glycinin (11S). The 7S subunit mRNA  
appeared several days before the 11S mRNA. Furthermore, while  
members of the glycinin gene family were all activated  
simultaneously (Nielsen et al. (1989) Plant Cell 1, 313),  
10 members of the  $\beta$ -conglycinin gene family were differentially  
regulated (Barker et al. (1988) Proc. Natl. Acad. Sci. USA 85,  
458; Chen et al. (1989) Dev. Genet. 10, 112). Each of these  
genes contain a different array of cis-regulatory elements which  
confer differential expression patterns between, and within,  
15 these gene families.

Helianthinin is the major 11S globulin seed storage  
protein of sunflower (Helianthus annuus). Helianthinin  
expression, like that of other seed storage proteins, is tissue-  
specific and under developmental control. However, the  
20 helianthin regulatory elements which confer such specificity  
have heretofore never been identified. Helianthinin mRNA is  
first detected in embryos 7 days post flowering (DPF) with  
maximum levels of mRNA reached at 12-15 DPF, after which the  
level of helianthinin transcripts begins to decline. In mature  
25 seeds or in germinating seedlings helianthinin transcripts are  
absent. Helianthinin polypeptide accumulation is rapid from 7  
DPF through 19 DPF but slows as the seed reaches later  
maturation stages (Allen et al. (1985) Plant Mol. Biol. 5, 165).

Helianthinin, like most seed proteins, is encoded by  
30 a small gene family. At least two divergent subfamilies are  
known, and are designated Ha2 and Ha10. Two clones, HaG3-A and  
HaG3-D, representing non-allelic members of the Ha2 subfamily,  
have been isolated and partially characterized (Vonder Haar et

1 al. (1988) Gene 74, 433). However, a detailed analysis of the  
regulatory elements of these or any other helianthinin genes had  
not been known until now.

5 It has been found in accordance with the present  
invention that regulatory elements from helianthinin genes can  
direct seed-specific gene expression, root-specific gene  
expression, abscisic acid-responsive gene expression, and/or  
temporally-altered gene expression. These regulatory elements  
enable the controlled expression of specific gene products in  
10 transgenic plants. The present invention provides greater  
control of gene expression in transgenic plants, thus allowing  
improved seed quality, improved tolerance to environmental  
conditions such as drought, and better control of herbicide  
resistance genes.

15 The present invention is directed to the 5' regulatory  
region of a helianthinin gene. This region is herein referred  
to as the upstream regulatory ensemble (URE), and is useful in  
directing the expression of heterologous proteins. The URE  
consists of multiple regulatory elements which confer distinct  
20 regulated expression patterns when linked to the coding regions  
of heterologous genes which are expressed in transgenic plants.

In particular, the present invention provides isolated  
DNA containing helianthinin regulatory elements which direct  
seed-specific gene expression, root-specific gene expression,  
25 abscisic acid (ABA)-responsive gene expression and/or  
temporally-altered gene expression.

Another aspect of this invention is directed to  
chimeric plant genes containing these regulatory elements. The  
regulatory elements are operably linked to the coding sequence  
of a heterologous gene such that the regulatory element is  
30 capable of controlling expression of the product encoded by the  
heterologous gene. If necessary, additional promoter elements  
or parts of these elements are included in the chimeric gene

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1 constructs. Plant transformation vectors comprising the  
chimeric genes of the present invention are also provided, as  
are plant cells transformed by these vectors, and plants and  
their progeny containing the chimeric genes.

5 In yet another aspect of this invention, a method is  
provided for producing a plant with improved seed-lipid quality.  
Chimeric genes are constructed according to the present  
invention in which a regulatory element directing seed-  
specific expression is linked to the coding region of a gene  
10 encoding a lipid metabolism enzyme. When plant cells are  
transformed with this chimeric gene, plants with improved seed  
lipid-quality can be regenerated.

A further aspect of the present invention provides a  
method for producing a herbicide-resistant plant. In accordance  
15 with the present invention, for example, chimeric genes are  
constructed in which a root-specific regulatory element directs  
the expression of herbicide-resistance gene. Plant cells are  
transformed with this chimeric gene to regenerate herbicide-  
resistant plants.

20 Fig. 1 depicts the nucleotide sequence of the URE of  
helianthinin gene HaG3-A. Nucleotide numbers -2377 to +24 of  
Fig. 1 correspond to nucleotide numbers 1 to 2401 of SEQ ID  
NO:1.

25 Fig. 2 depicts the nucleotide sequence of part of the  
URE of helianthinin gene HaG3-D. Nucleotide numbers -2457 to -  
726 of Fig. 2 correspond to nucleotide numbers 1 to 1732 of SEQ  
ID NO:2.

30 Fig. 3 represents the nucleotide sequence of part of  
the URE of helianthinin gene HaG3-D. Nucleotide numbers -725  
to -322 of Fig. 3 correspond to nucleotide numbers 1 to 404 of  
SEQ ID NO:3. In the helianthinin gene HaG3-D, the nucleotide  
sequence of Fig. 3 is immediately downstream (3') of the  
sequence of Fig. 2.

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1           Fig. 4 depicts the HaG3-A FL/GUS construction and the control constructions pBI121.1 and pBI101.1.

5           Fig. 5 depicts a restriction map of helianthinin genomic clones HaG3-A and HaG3-D and the restriction fragments used to construct the parental plasmids.

          Fig. 6 depicts the HaG3-A and HaG3-D derivative constructions in relation to the full length construction.

10          Fig. 7 demonstrates histochemical localization of GUS activity in transgenic seedlings containing the HaG3-D-N and HaG3-A-SB/R constructions. A: HaG3-D-404N, 8 days post-imbibition (DPI); B: HaG3-A-SB/R, 8 DPI; C: HaG3-D-404N, 14 DPI; D: HaG3-A-SB/R, 14 DPI; E: HaG3-A-SB/R, 8 DPI; F: HaG3-A-SB/R, 6 DPI.

15          Fig. 8 graphically illustrates the induction of GUS activity in transgenic tobacco leaves containing HaG3-D-404N during progressive desiccation and subsequent recovery from water deficit.

          Fig. 9 is a graph depicting ABA induction of GUS expression in leaves of tobacco containing HaG3-D-404N.

20          The present invention comprises cis-regulatory elements of the upstream regulatory ensemble (URE) of sunflower helianthinin genes. These cis-regulatory elements are discrete regions of the URE that confer regulated expression upon the gene under their control. In particular, this invention provides isolated nucleic acid containing at least one regulatory element from a helianthinin gene which directs at least one of the following: seed-specific gene expression, root-specific gene expression, ABA-responsive gene expression or temporally-altered gene expression. Any helianthinin gene can provide the regulatory elements, including Ha2 and Ha10 genes, which represent two divergent helianthinin gene subfamilies. In a preferred embodiment, the helianthinin genes are HaG3-A and HaG3-D, which are members of the Ha2 subfamily.

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1           One of the subject regulatory elements directs seed-specific expression. A seed-specific regulatory element represents a particular nucleotide sequence that is capable of causing the expression of the gene under its control to occur in the seed, i.e. for the gene produced to be detected in the seed. Expression that is seed-specific may be in any part of the seed, e.g., but not limited to, the cotyledons and embryonic axis of the embryo and to the endosperm. No gene expression is detected in seedlings or somatic tissues of the adult plant for genes under seed-specific control.

10           To identify regulatory elements that direct seed-specific expression, a deletion analysis of the entire URE of a helianthinin gene can be performed. In a deletion analysis, nucleotides are successively removed from the entire URE, and the resulting fragments are ligated to the coding sequence of a reporter gene or other heterologous gene. The constructs are then analyzed for their ability to direct seed-specific expression by detecting the presence of the reporter gene product in seed tissues and not in other tissues. The seed-specific elements which have been identified can also be modified, e.g. by site-directed mutagenesis. The modified regulatory elements can then be assayed for their ability to direct seed-specific expression, thereby identifying alternative sequences that confer seed-specificity. These techniques for identifying regulatory elements are applicable to all helianthinin genes. For example, in a preferred embodiment an analysis of the URE of the helianthinin HaG3-A gene indicates that seed-specific regulatory elements are provided by nucleotides 851 to 2401, and by nucleotides 1 to 2401 of SEQ ID NO:1.

30           Other regulatory elements provided by the present invention provide root-specific expression. Root-specific expression is of particular interest and importance. Normally

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1 the sunflower helianthinin gene is expressed only in seeds.  
When particular regions of the helianthinin URE are isolated  
from the entire URE in accordance with the present invention,  
expression is exclusively localized to plant roots. A root-  
5 specific regulatory element represents a particular nucleotide  
sequence that is capable of causing the expression of the gene  
under its control to occur in plant roots and not in other plant  
tissues. Regulatory elements that direct root-specific  
expression are identified by analyzing fragments of a  
10 helianthinin URE for their ability to confer root-specific  
expression as described above for the identification of seed-  
specific regulatory elements except expression is detected in  
root tissues. Modifications of the nucleotide sequences that  
permit root-specific expression are also identified as described  
15 above. Root-specific regulatory elements from any helianthinin  
gene can be identified by such techniques. For example, in a  
preferred embodiment, an analysis of the URE of the helianthinin  
HaG3-A gene indicates that nucleotides 1 to 1639 and nucleotides  
851 to 1639 of SEQ ID NO:1 represent root-specific regulatory  
20 elements.

Helianthinin expression is under strict temporal  
control, with mRNA first detected at 12 DPF. Accordingly, it  
has been discovered that cis-regulatory elements exist which  
confer temporally-altered gene expression which is detectable  
25 as early as about 4 DPF.

To identify regulatory elements that confer  
temporally-altered gene expression, a deletion analysis of the  
entire URE of a helianthinin gene can be performed. Fragments  
of the URE are linked to the coding sequence of a heterologous  
30 gene and the resulting chimeric construction is used to  
transform plants. Seeds from transformed plants are staged by  
days post flowering, and the staged seeds are assayed to detect  
the expression of the heterologous gene. Elements that direct  
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1 expression of the heterologous gene before about 10 DPF are  
identified as elements that confer temporally-altered  
expression. Modifications of the nucleotide sequences of such  
elements that confer the desired phenotype can be identified as  
5 described above. These techniques for identification of  
regulatory elements that confer temporally-altered gene  
expression are applicable to all helianthinin genes. In a  
preferred embodiment, an analysis of the URE of the helianthinin  
gene HaG3-A indicates that elements that confer temporally-  
10 altered gene expression are provided by nucleotide 1 to 851 and  
1639 to 2303 of SEQ ID NO:1.

Another aspect of the present invention is directed  
to regions of the URE of helianthinin that confer abscisic acid  
(ABA)-responsive gene expression. An ABA-responsive element  
15 represents a particular nucleotide sequence that is capable of  
causing the gene under its control to be expressed in response  
to ABA. Expression of the gene under the control of the ABA-  
responsive element can be induced by treatment with ABA, or by  
external stimuli that are known to result in the initiation of  
20 ABA biosynthesis. For example, ABA biosynthesis is initiated  
as a result of loss of turgor caused by environmental stresses  
including water-deficit, water-stress and salt-stress (reviewed  
in Zeevaart et al. (1988) Annu. Rev. Plant Physiol. 39, 439).  
Levels of ABA also increase in response to wounding, (Peña-  
25 Cortes et al. (1989) Proc. Natl. Acad Sci. USA 86, 9851).  
ABA-responsive elements are identified as described above for  
the identification of other regulatory elements. For example,  
deletion analysis can be used to identify nucleotide sequences  
of any helianthinin gene that induce the expression of a gene  
30 under its control in response to ABA. Such sequences can be  
modified as described above, and assayed to identify alternative  
sequences that confer ABA-responsive expression. In one  
preferred embodiment, an analysis of the URE of the helianthinin

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1 HaG3-A gene indicates that nucleotides 1 to 2401 of SEQ ID NO:1  
provide an element that confers ABA-responsive expression in  
seeds. In another preferred embodiment, nucleotides 851 to 1639  
or 1639 to 2303 of SEQ ID NO:1 provide an element that confers  
5 ABA-responsive expression in leaves of adult plants. In yet  
another preferred embodiment, an analysis of the URE of the  
helianthinin HaG3-D gene indicates that nucleotides 1 to 404 of  
SEQ ID NO:3 confer ABA-responsive expression in non-embryonic  
tissues of plants.

10 Accordingly, ABA-responsive elements have utility in  
that specific environmental cues can initiate ABA biosynthesis,  
and further induce expression of genes under the control of an  
ABA-responsive element. Expression of heterologous genes driven  
by the ABA-responsive elements of the helianthinin URE is not  
15 restricted to seeds, but is also observed in leaves of adult  
plants and in tissues of seedlings.

An isolated nucleic acid encoding the upstream  
regulatory ensemble of a helianthinin gene can be provided as  
follows. Helianthinin recombinant genomic clones are isolated  
20 by screening a sunflower genomic DNA library with a cDNA  
recombinant representing helianthinin mRNA (Vonder Haar (1988)  
Gene 74, 433). Methods considered useful in obtaining  
helianthinin genomic recombinant DNA are contained in Sambrook  
et al., 1989, in Molecular Cloning: A Laboratory Manual, Cold  
25 Spring Harbor, NY, for example, or any of the myriad of  
laboratory manuals on recombinant DNA technology that are widely  
available. To determine nucleotide sequences, a multitude of  
techniques are available and known to the ordinarily skilled  
artisan. For example, restriction fragments containing a  
30 helianthinin URE can be subcloned into the polylinker site of  
a sequencing vector such as pBluescript (Stratagene). These  
pBluescript subclones can then be sequenced by the double-strand  
dideoxy method (Chen and Seeburg (1985) DNA 4, 165).

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1           The nucleotide sequence for DNA encoding the URE of  
helianthinin gene clone HaG3A is shown in Fig. 1 and presented  
as SEQ ID NO:1. Similarly, the nucleotide sequence for DNA  
encoding a region of the URE of helianthinin clone HaG3D is  
5 shown in Fig. 2 and presented as nucleotide sequence SEQ ID  
NO:2. The UREs of other helianthinin genes can be obtained by  
the same strategy. Alternatively, clones representative of  
other members of the helianthinin gene family can be obtained  
by using the HaG3A or HaG3D coding or URE sequences of the  
10 present invention as hybridization probes to screen a  
helianthinin genomic library and identify the additional  
helianthinin genes.

The identification of cis-regulatory sequences that  
direct temporal, tissue-specific and ABA-responsive regulation  
15 can be accomplished by transcriptional fusions of specific  
sequences with the coding sequence of a heterologous gene,  
transfer of the chimeric gene into an appropriate host, and  
detection of the expression of the heterologous gene. The assay  
used to detect expression depends upon the nature of the  
heterologous sequence. For example, reporter genes, exemplified  
20 by chloramphenicol acetyl transferase and  $\beta$ -glucuronidase (GUS),  
are commonly used to assess transcriptional and translational  
competence of chimeric constructions. Standard assays are  
available to sensitively detect the reporter enzyme in a  
transgenic organism. The  $\beta$ -glucuronidase (GUS) gene is useful  
25 as a reporter of promoter activity in transgenic tobacco plants  
because of the high stability of the enzyme in tobacco cells,  
the lack of intrinsic  $\beta$ -glucuronidase activity in higher plants  
and availability of a qualitative fluorimetric assay and a  
histochemical localization technique. Jefferson *et al.* [(1987)  
30 EMBO J, 6, 3901]] have established standard procedures for  
biochemical and histochemical detection of GUS activity in plant  
tissues. Biochemical assays are performed by mixing plant

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1 tissue lysates with 4-methylumbelliferyl- $\beta$ -D-glucuronide, a  
fluorimetric substrate for GUS, incubating one hour at 37°C, and  
then measuring the fluorescence of the resulting 4-methyl-  
5 umbelliferone. Histochemical localization for GUS activity is  
determined by incubating plant tissue samples in 5-bromo-4-  
chloro-3-indolyl-glucuronide (X-Gluc) for 18 hours at 37°C, and  
observing the staining pattern of X-Gluc. The construction of  
such chimeric genes allows definition of specific regulatory  
sequences required for regulation of expression, and  
10 demonstrates that these sequences can direct expression of  
heterologous genes in the manner under analysis.

Another aspect of the present invention is directed  
to a chimeric plant gene containing a regulatory element from  
a helianthinin gene which directs seed-specific gene expression,  
15 root-specific gene expression, ABA-responsive gene expression  
or temporally-altered gene expression linked to the coding  
sequence of a heterologous gene such that the regulatory element  
is capable of controlling expression of the product encoded by  
the heterologous gene. The heterologous gene can be any gene  
20 other than helianthinin. If necessary, additional promoter  
elements or parts of these elements sufficient to cause  
expression resulting in production of an effective amount of the  
polypeptide encoded by the heterologous gene are included in the  
chimeric constructs.

25 Accordingly, the present invention provides chimeric  
genes comprising regions of the helianthinin URE that confer  
seed-specific expression in accordance with this invention which  
are linked to a sequence encoding a lipid metabolism enzyme such  
as a desaturase. In a preferred embodiment, the regions of the  
30 URE comprise nucleotides 851 to 2401 or 1 to 2401 of HaG3-A as  
shown in SEQ ID NO:1. Any modification of these sequences which  
confers seed-specific expression is contemplated. Seeds  
accumulate and store proteins and lipids, both of significant

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1 agronomic importance. Because elements of the helianthinin URE  
can direct high, regulated expression in developing seeds, these  
elements have utility in improving seed lipid and/or protein  
quality. These elements are useful in regulating expression of  
5 genes encoding lipid metabolism enzymes, such as those involved  
in elongation and desaturation of fatty acids, and/or proteins,  
especially those with high lysine and methionine content.  
Chimeric genes containing these elements can be used to provide  
transgenic plant lines that accumulate and store significant  
10 amounts of specific classes of lipids and/or proteins.

In another aspect of the present invention chimeric  
genes are provided which have a region of the URE of  
helianthinin that confers root-specific expression fused to a  
heterologous gene. This construction confers expression  
15 spatially distinct from "normal" helianthinin expression in that  
the heterologous gene is expressed exclusively in plant roots.  
In other words, when a specific sequence is removed from the  
context of the entire URE, tissue-specific regulation is  
altered. In a preferred embodiment, the region of the HaG3-A  
20 URE comprises 1 to 1639 or 851 to 1639 of SEQ ID NO:1 and is  
fused in reverse orientation to the promoter although these  
elements function in either orientation. In another preferred  
embodiment the sequence providing herbicide resistance is at  
least part of the aroA gene. Any modification of these  
25 sequences which confers root-specific expression is  
contemplated.

Of particular importance is the use of these chimeric  
constructions to confer herbicide resistance. Since most  
herbicides do not distinguish between weeds and crop plants, the  
30 engineering of herbicide-resistant crop plants is of  
considerable agronomic importance in that it allows the use of  
broad-spectrum herbicides. Accordingly, the present invention  
provides chimeric genes comprising elements of a helianthinin

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1 URE that confer root-specific expression fused to at least part  
of a promoter that functions in plants and further fused to at  
least part of the aroA gene or a sequence encoding a polypeptide  
conferring herbicide resistance. Polypeptides that confer  
5 resistance to glyphosate and related inhibitors of 5-  
enolpyruvylshikimic acid-3-phosphate synthase (EPSP synthase),  
sulfonyleureas, imidazolinones and inhibitors of acetolactase  
synthase (ALS) and acetoxy acid synthase (AHS) are  
contemplated. In a preferred embodiment the regions of the URE  
10 are 1 to 1639 or 851 to 1639 of HaG3-A, as shown in SEQ ID NO:1  
and are fused in reverse orientation to the promoter. Any  
modification of these sequences which confers root-specific  
expression is contemplated.

15 In another aspect of the present invention chimeric  
genes are provided comprising elements of the URE of  
helianthinin that confer temporally-altered expression fused in  
forward or reverse orientation to at least part of a promoter  
that functions in plants and further linked to the coding region  
of a heterologous gene. In a preferred embodiment the elements  
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1 of the URE are nucleotides 1 to 851 or 1639 to 2303 of HaG3-A,  
as shown in SEQ ID NO:1. Any modification of these sequences  
that confers temporally altered gene expression is contemplated.

Chimeric genes are provided comprising elements of the  
5 URE of a helianthinin that confer ABA-responsive expression  
optionally fused in forward or reverse orientation to at least  
part of a promoter that functions in plants further fused to a  
heterologous gene. In a preferred embodiment the element of the  
URE comprises 851 to 1639 or 1639 to 2303 of HaG3-A, as shown  
10 in SEQ ID NO:1, or nucleotides 1 to 404 of HaG3-D, as shown in  
SEQ ID NO:3. Of particular importance is the use of constructs  
that confer ABA-responsive expression to provide plants with  
improved tolerance to water stress.

The chimeric genes of the present invention are  
15 constructed by fusing a 5' flanking sequence of a helianthinin  
genomic DNA to the coding sequence of a heterologous gene. The  
juxtaposition of these sequences can be accomplished in a  
variety of ways. In a preferred embodiment the order of  
sequences, from 5' to 3', is a helianthinin upstream regulatory  
20 region, a promoter region, a coding sequence, and a  
polyadenylation site.

Standard techniques for construction of such chimeric  
genes are well known to those of ordinary skill in the art and  
can be found in references such as Sambrook et al. (1989). A  
25 variety of strategies are available for ligating fragments of  
DNA, the choice of which depends on the nature of the termini  
of the DNA fragments. One of ordinary skill in the art  
recognizes that in order for the heterologous gene to be  
expressed, the construction requires promoter elements and  
30 signals for efficient polyadenylation of the transcript.  
Accordingly, the 5' helianthinin URE regions that contain the  
promoter sequences known as CAAT and TATA boxes can be fused  
directly to a promoterless heterologous coding sequence.

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1 Alternatively, the helianthinin URE regions that do not contain  
the CAAT and TATA boxes can be joined to a DNA fragment encoding  
a promoter that functions in plants. Plant promoters can be  
5 obtained commercially, or can be chemically synthesized based  
on their published sequences. An example of such a fragment is  
the truncated cauliflower mosaic virus 35S promoter, which  
retains its CAAT and TATA boxes. Other representative promoters  
include the nopaline synthase and ribulose 1,5 biphosphate  
10 carboxylase promoters. The promoter fragment is further linked  
to the heterologous coding sequence. The 3' end of the coding  
sequence is fused to a polyadenylation site exemplified by, but  
not limited to, the nopaline synthase polyadenylation site.  
Furthermore, intermediate plant transformation vectors are  
15 available that contain one or more of these polyadenylation  
sites bordered by sequences required for plant transformation.  
The elements of the helianthinin URE and the heterologous coding  
sequences of the present invention can be subcloned into the  
polylinker site of a plant transformation vector to provide the  
chimeric genes.

20 The 5' flanking elements of the present invention can  
be derived from restriction endonuclease or exonuclease  
digestion of a helianthinin genomic clone. The restriction  
fragments that contain the helianthinin CAAT and TATA boxes are  
ligated in a forward orientation to a promoterless heterologous  
25 gene such as the coding sequence of  $\beta$ -glucuronidase (GUS). The  
skilled artisan will recognize that the 5' helianthinin  
regulatory sequences can be provided by other means, for example  
chemical or enzymatic synthesis. The heterologous product can  
be the coding sequence of any gene that can be expressed in such  
30 a construction. Such embodiments are contemplated by the  
present invention. The 3' end of the coding sequence is  
optionally fused to a polyadenylation site, exemplified by, but  
not limited to, the nopaline synthase polyadenylation site, or

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1 the octopine T-DNA gene 7 polyadenylation site. Alternatively,  
the polyadenylation site can be provided by the heterologous  
gene.

5 The 5' helianthinin regulatory elements that do not  
contain the TATA box can be linked in forward or reverse  
orientation to at least part of a plant promoter sequence, i.e.  
a plant promoter sequence containing at least the CAAT and TATA  
sequences. In a preferred embodiment, this promoter is a  
10 truncated cauliflower mosaic virus (CaMV) 35S promoter. The  
resulting chimeric complex can be ligated to a heterologous  
coding sequence and a polyadenylation sequence.

To provide regulated expression of the heterologous  
genes, plants are transformed with the chimeric gene  
constructions of this invention. Gene transfer is well known  
15 in the art as a method to express heterologous genes in  
transgenic plants. The tobacco plant is most commonly used as  
a host because it is easily regenerated, yields a large number  
of developing seeds per plant, and can be transformed at a high  
frequency with Agrobacterium-derived Ti plasmid vectors (Klee,  
20 et al. (1987) Annu. Rev. Plant Physiol. 38, 467).  
Dicotyledenous plants including cotton, oil seed rape and  
soybean are preferred as transgenic hosts. However, one of  
ordinary skill in the art will recognize that any plant that can  
be effectively transformed and regenerated can be used as a  
25 transgenic host in the present invention.

A variety of transformation methods are known. The  
chimeric genes can be introduced into plants by a leaf disk  
transformation-regeneration procedure as described by Horsch et  
al. (1985) Science 227, 1229). Other methods of transformation,  
30 such as protoplast culture (Horsch et al., (1984) Science 223,  
496; DeBlock et al. (1984) EMBO J. 2, 2143; Barton et al. (1983)  
Cell 32, 1033) or transformation of stem or root explants in  
vitro (Zambryski et al. (1983) EMBO J. 2, 2143; Barton et al.

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1 (1983) Cell 32, 1033) can also be used and are within the scope  
of this invention. In a preferred embodiment plants are  
transformed with Agrobacterium-derived vectors. However, other  
5 methods are available to insert the chimeric genes of the  
present invention into plant cells. Such alternative methods  
include biolistic approaches (Klein et al. (1987) Nature 327,  
70) electroporation, chemically-induced DNA uptake, and use of  
viruses or pollen as vectors.

When necessary for the transformation method, the  
10 chimeric genes of the present invention can be inserted into a  
plant transformation vector, e.g. the binary vector described  
by Bevan (1984). Plant transformation vectors can be derived  
by modifying the natural gene transfer system of Agrobacterium  
tumefaciens. The natural system comprises large Ti (tumor-  
15 inducing)-plasmids containing a large segment, known as T-DNA,  
which is transferred to transformed plants. Another segment of  
the Ti plasmid, the vir region, is responsible for T-DNA  
transfer. The T-DNA region is bordered by terminal repeats.  
In the modified binary vectors the tumor-inducing genes have  
20 been deleted and the functions of the vir region are utilized  
to transfer foreign DNA bordered by the T-DNA border sequences.  
The T-region also contains a selectable marker for antibiotic  
resistance, and a multiple cloning site for inserting sequences  
for transfer. Such engineered strains are known as "disarmed"  
25 A. tumefaciens strains, and allow the efficient transformation  
of sequences bordered by the T-region into the nuclear genomes  
of plants.

Surface-sterilized leaf disks are inoculated with the  
"disarmed" foreign DNA-containing A. tumefaciens, cultured for  
30 two days, and then transferred to antibiotic-containing medium.  
Transformed shoots are selected after rooting in medium  
containing the appropriate antibiotic, and transferred to soil.

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1 Transgenic plants are self-pollinated and seeds from these plants are collected and grown on antibiotic-containing medium.

Expression of a heterologous or reporter gene in developing seeds, young seedlings and mature plants can be  
5 monitored by immunological, histochemical or activity assays.

As discussed herein, the choice of an assay for expression of the chimeric gene depends upon the nature of the heterologous coding region. For example, Northern analysis can be used to assess transcription if appropriate nucleotide probes  
10 are available. If antibodies to the polypeptide encoded by the heterologous gene are available, Western analysis and immunohistochemical localization can be used to assess the production and localization of the polypeptide. Depending upon the heterologous gene, appropriate biochemical assays can be  
15 used. For example, acetyltransferases are detected by measuring acetylation of a standard substrate. The expression of an herbicide-resistance gene can be detected by determining the herbicide resistance of the transgenic plant.

Another aspect of the present invention provides  
20 transgenic plants or progeny of these plants containing the chimeric genes of the invention. Both monocotyledenous and dicotyledenous plants are contemplated. Plant cells are transformed with the chimeric genes by any of the plant transformation methods described above. The transformed plant  
25 cell, usually in a callus culture or leaf disk, is regenerated into a complete transgenic plant by methods well-known to one of ordinary skill in the art (e.g. Horsch et al. (1985) Science 227, 1129). In a preferred embodiment, the transgenic plant is cotton, oil seed rape, maize, tobacco, or soybean. Since  
30 progeny of transformed plants inherit the chimeric genes, seeds or cuttings from transformed plants are used to maintain the transgenic plant line.

1           The instant invention also provides a method for  
2           producing a plant with improved seed lipid quality. This method  
3           comprises transforming a plant cell with a vector containing a  
4           chimeric gene comprising a seed-specific regulatory element  
5           linked to the coding sequence of a lipid metabolism enzyme such  
6           as a desaturase, and selecting for a plant with the desired  
7           characteristics. In a preferred embodiment the regulatory  
8           element is provided by nucleotides 1 to 2401 or 851 to 2401 of  
9           the URE of HaG3A as shown in SEQ ID NO:1. The transformed plant  
10          cells are regenerated into plants with improved seed lipid  
11          quality.

12          Another aspect of the present invention provides a  
13          method for producing a plant with improved seed protein quality.  
14          This method comprises transforming a plant cell with a vector  
15          containing a chimeric gene comprising a seed-specific regulatory  
16          element linked to the coding sequence of a seed storage protein  
17          with a high content of lysine and/or methionine residues, and  
18          selecting for a plant with the desired characteristic. In a  
19          preferred embodiment the regulatory element is provided by  
20          nucleotides 1 to 2401 or 851 to 2401 of the URE of HaG3-A as  
21          shown in SEQ ID NO:1. The transformed plant cells are  
22          regenerated into plants with improved seed protein quality.

23          Another aspect of the present invention provides a  
24          method for producing a herbicide-resistant plant. Plant cells  
25          are transformed with a vector containing a chimeric gene  
26          comprising a root-specific regulatory element linked to the  
27          coding sequence of a herbicide resistance gene such as a  
28          glyphosate resistance gene and then plants with the desired  
29          herbicide resistance are selected. Selected plants are those  
30          which survive a herbicide treatment which kills untransformed  
31          plants of the same kind under the same conditions. In a  
32          preferred embodiment, the regulatory element is provided by  
33          nucleotides 1 to 1639 or 851 to 1639 of the URE of HaG3-A as  
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1 shown in SEQ ID NO:1, and the heterologous sequence is provided  
by a gene encoding EPSP synthase, acetolactase synthase, or  
acetohydroxy acid synthase. The transformed plant cells are  
5 regenerated into herbicide-resistant plants. In a preferred  
embodiment, plants are transformed by the vector pRPA-ML-803,  
which contains the root-specific regulatory element comprising  
nucleotides 851 to 1639 of HaG3-A and the aroA herbicide-  
resistance gene.

10 The following examples further illustrate the  
invention.

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EXAMPLE 1General Methods

The nucleotide sequences referred to in the following examples are numbered according to Fig. 1-3.

GUS Reporter Gene Constructions

The general purpose GUS reporter cassettes used throughout the examples have been described previously (Jefferson et al. (1987) EMBO J. 6, 3901). Briefly, the coding region of GUS was ligated 5' of the nopaline synthase polyadenylation site in the polylinker site of the A. tumefaciens-derived vector pBIN19 (Bevan (1984) Nucleic Acids Res. 12, 8711). The vector pBIN19 contains the left and right borders of T-DNA necessary for plant transformation, and a kanamycin resistance gene. The resulting construction, pBI101.1, is depicted in Figure 4. Unique restriction sites upstream of the AUG initiation codon of GUS allow the insertion of promoter DNA fragments.

The CaMV 35S promoter was ligated into the HindIII and BamHI sites of pBI101.1 to create pBI121.1, depicted in Fig. 4. To create pBI120, the CaMV 35S promoter was truncated at an EcoRV site at -90 (leaving the CAAT and TATA boxes) and cloned into the polylinker site of pBI101.1.

Table 1 describes the parental plasmids and derivative constructions. HaG3-A-FL and the control constructions pBI121.1 and pBI101.1 are depicted in Figure 4. Figure 5 shows the restriction fragments of genomic clones HaG3-A and HaG3-D used to construct parental plasmids. Figure 6 shows the derivative constructions schematically in relation to the full length construction.

The HaG3-A/GUS constructions represent large overlapping fragments that span the full length regulatory region (-2377 to +24 of Fig. 1). The 3' ends of several



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1 constructions were derived from exonuclease III digestions of  
a 2.8 kb HaG3-A fragment in pBluescript (Stratagene) [pHaG3-A-  
2.8 (BamHI-PstI), Table 1]. These deletions are shown at the  
top of Figure 4. The first deletion, pHaG3-A-2.4, contains the  
5 HaG3-A CAAT and TATA boxes with its 3' end at -75. Fragments  
that contained the HaG3-A CAAT and TATA boxes were ligated in  
forward orientation into the promoterless GUS cassette pBI101.1.  
Fragments that did not contain the HaG3-A TATA box were ligated  
in both orientations upstream of the truncated CaMV 35S promoter  
10 of pBI120. These fragments were subcloned into the appropriate  
GUS cassette. Constructions are named according to their end  
sites followed by an F, indicating forward orientation; R,  
indicating reverse orientation. Arrows indicate the orientation  
of the fragment with respect to the GUS coding region (Fig. 4).  
15 The HaG3-D/GUS constructions contain a 404 bp fragment (Sall-  
HpaI) in both orientations: Normal (N) and Inverse (I). The  
accuracy and orientation of each construction was confirmed by  
double-stranded dideoxy sequencing (Chen and Seeburg, 1985)  
using primers to regions in the GUS cassettes (Advanced DNA  
20 Technologies Lab, Texas A&M University).

#### Plant Transformation

The BIN-19 based plasmid constructions were used to  
transform tobacco (Nicotiana tabacum cv. Xanthi) according to  
standard procedures (Horsch et al. 1985) except that initial  
25 transformants were selected on 50 µg kanamycin/ml and then were  
transferred to 100 µg/ml kanamycin. Plants were self-  
pollinated, and seeds were germinated on kanamycin (400 g/ml)  
to identify transformants, since the BIN-19 based constructions  
contain the neomycin phosphotransferase gene (NPTII), which  
30 confers resistance to the toxic antibiotic kanamycin. The copy  
number of each GUS construction integrated into the tobacco  
genome was estimated for each transformant by segregation  
frequencies of the NPTII gene. Most of the transformants

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1 contained only one segregating locus of the construction.  
2 Filial, homozygous plants were used where indicated. Transgenic  
3 plants representing all of the test constructions were obtained  
4 except for the reverse construction of H- 2. Transgenic plants  
5 were maintained in Conviron chambers: 16h light:8h dark, 24°C,  
6 70-80% relative humidity. All plants were watered on a strict  
7 schedule to prevent desiccation prior to testing.

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TABLE 1

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<u>Construction</u>		<u>Description</u>
<u>Parental Plasmids</u>		
5	pBI101.1	Bin 19-derived promoterless GUS reporter gene cassette.
	pBI121.1	CaMV 35S promoter fused to GUS cassette in pBI 101.1.
10	pBI120	CaMV 35S promoter truncated at EcoRV site, leaving CAAT and TATA boxes, fused to GUS coding region.
	pHaG3-A-2.8	2.8 kb BamIH-PstI fragment of HaG3-A in pBluescript; contains 2.4 kb upstream of HaG3-A coding region and 0.38 kb downstream of transcription start site; used to generate exonuclease III deletions.
15	pHaG3-A-2.4	2.4 kb HaG3-A fragment generated from 3' exonuclease III digestion of pHaG3A-2.8 to +24; contains the HaG3-A CAAT and TATA boxes.
20	pHaG3-A-2.3	2.3 kb HaG3-A fragment generated from 3' exonuclease III digestion of pHaG3A-2.8 to -75; contains the HaG3-A CAAT box.

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1 Derivative Constructions

- HaG3-A-FL 2.4 kb insert of pHaG3A-2.4 fused to pBI101.1 in forward orientation
- 5 HaG3-A-HS/F 0.85 kb BamH1-SalI fragment from pHaG3A-2.3  
-HS/R cloned in forward and reverse orientation with respect to the truncated CaMV 35S promoter of pBI120.
- HAG3-A-HS/R 0.85 kb excised as a SacI fragment from HaG3A-HS/F and cloned in reverse orientation with respect to the truncated CaMV 35S promoter pBI120.
- 10 HaG3-A-HB/F 1.6 kb BamH1-BalI fragment from pHaG3A-2.3  
-HB/R cloned in forward and reverse orientations with respect to the truncated CaMV 35S promoter pBI120.
- 15 HaG3-A-S 2/F 0.6 kb SalI-BalI from HaG3-A cloned in forward and reverse orientations with respect to the truncated CaMV 35S promoter of pBI120;  
-S 2/R constructed by deleting SalI-BamH1 fragment from HaG3-A-HB/F and HaG3-A-HB/R, respectively.
- HaG3-A-S 2/F 1.4 kb SalI fragment from pHaG3-A-2.3 cloned in forward and reverse orientation with respect to the truncated CaMV 35S promoter in pBI120.  
-S 2/R
- 20 HaG3-A-B 2/F 0.66 kb BalI SalI fragment from pHaG3-A-2.3  
-B 2/R cloned in forward and reverse orientation with respect to the truncated CaMV 35S promoter in pBI120.
- 25 HaG3-A-H 2 2.3 kb insert from pHaG3-A-2.3 cloned in forward orientation with respect to the truncated CaMV 35S promoter of pBI120.
- HaG3-A-S 1 1.5 kb SalI fragment from pHaG3-A-2.4 cloned in forward orientation with respect to pBI101.1.
- 30 HaG3-D-404N 0.4 kb SalI-HpaI fragment form HaG3-D cloned in forward and reverse orientation with respect to the truncated CaMV 35S promoter in pBI120.  
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EXAMPLE 2Biochemical Detection of GUS Activity:  
Seed-Specific and Root-Specific Expression

GUS activity was determined in embryonic and non-embryonic tissues of transgenic tobacco containing each construction of Table 1. The standard procedures of Jefferson et al. (1987) were followed.

Plant tissue was ground in extraction buffer (50 mM NaPO<sub>4</sub>, 10 mM EDTA, 0.1% Sarkosyl, 0.1% Triton X-100 and 10 mM β-mercaptoethanol). After centrifugation of the lysate, the supernatant was removed to a fresh tube and dispensed in 100 μl aliquots. An equal volume of 2 mM 4-methylumbelliferyl-β-D-glucuronide in extraction buffer was added and allowed to incubate at 37°C for 1 h. Reactions were stopped with 0.8 ml Na<sub>2</sub>CO<sub>3</sub> (0.2 M). The fluorescence of the resulting 4-methylumbelliferone (4-MU) was determined with a Hoeffer TKO-100 minifluorometer as described (Jefferson et al. 1987). GUS activity is expressed in picomoles 4-MU per unit mass total protein sample per minute.

Cotyledons, hypocotyls, leaves, and roots from transgenic seedlings, ranging from 18 to 20 days post-inbibition (DPI), containing various sequence elements of HaG3-A (summarized in Fig. 4) driving GUS expression were assayed for activity. Results are provided in Table 2. All constructions containing some portion of the URE of the helianthinin genes HaG3-A and HaG3-D conferred GUS activity in transgenic tobacco seeds. The full length regulatory region (FL) and fragments derived from this region, as well as the HaG3-D/GUS constructions, all conferred significant GUS activity in mature seeds when compared with the GUS expression driven by the intact CaMV 35S promoter complex (pBI121). However, well-defined seed-specific expression was only obtained with constructs including

1 the proximal upstream regions between -75 and +24 (cf. FL and  
S- 1). These two constructions containing nucleotides -2377 to  
+24 or -1527 to +24 demonstrated tissue-specific GUS expression  
5 with no detectable GUS activity in any tissues of transgenic  
seedlings. The FL construct, however, was expressed in mature  
seeds at sixfold higher levels compared to S- 1. GUS activity  
in tissues of seedlings containing the intact CaMV 35S promoter  
complex (pBI121) are included for comparison as well as the  
10 negative controls containing the truncated CaMV 35S promoter  
(pBI120) or no promoter (pBI101). Compared to expression in  
seeds there was little expression in leaves containing the same  
construction; on the other hand, most constructions, other than  
FL and S- 1, demonstrated significant expression in roots of  
transgenic seedlings.

15 The overall activity conferred by the intact CaMV 35S  
promoter complex was higher than that conferred by all other  
constructions in somatic tissue except in roots. In particular,  
roots of seedlings containing the HB/R (-2377 to -739) and SB/R  
(-1527 to -739) constructions showed levels of GUS activity 7  
20 to 8 times above that of roots expressing GUS under control of  
the intact CaMV 35S promoter.

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TABLE 2

Summary of GUS Expression in Embryonic and  
Non-Embryonic Tissues of 'Transgenic Tobacco'

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CONSTRUCTION <sup>1)</sup>		DEVELOPMENTAL PROFILE <sup>2)</sup>	GUS ACTIVITY (pmole 4MU/μg/min) <sup>3)</sup>			ABA Response <sup>4)</sup>
			SEEDS <sup>5)</sup>	LEAF <sup>6)</sup>	ROOT <sup>7)</sup>	
<u>HaG3-A</u>						
	FL	I	18.7±8.7	0	0	+
10	S- 1	I	3.4±1.1	0	0	ND
	F	III	17.1±15	0.45±0.05	36.6±2.2	-
	HS					
	R	ND <sup>8)</sup>	6.2±1.0	0.23±0.05	8.9±1.8	-
	F	II	14.8±5.2	0.95±0.13	29.9±2.2	+
15	HB					
	R	ND	13.1±6.9	0.25±0.05	75.4±3	-
	F	II	11.1±5.8	0	13.9±6.8	-
	SB					
	R	ND	12.1±5.8	0.34±0.05	90.5±9.9	+
	F	II	35.7±4.2	0	20.6±10.2	ND
20	S- 2					
	R	ND	21.0±15	0.45±0.08	38.8±1.2	+
	F	III	11.2±3.9	2.03±0.08	8.0±0.62	+
	B- 2					
	R	ND	7.2±2.3	4.05±0.10	3.9±0.3	+
25	H- 2 F	III	1.8±1.0	ND	1.8±0.3	ND
<u>HaG3-D</u>						
	N	III	9.2±2.9	0.07±0.01	6.8±0.5	+
	404					
	I	ND	9.2±3.9	2.03±0.05	12.9±2.8	+
30	<u>Controls</u>					
	pBI 101	ND	0	0	0	-
	pBI 120	ND	0	0	0	-
	pBI 121	ND	4.3±1.0	22.0±7.9	9.9±4.0	-

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1 TABLE 2 (continued)

- a Mature seeds and seedling tissues of transgenic tobacco containing constructions in Fig. 1 were assayed for GUS activity.
- 5 b Constructions are as shown in Fig. 1. Forward (F) and Reverse (R), Normal (N) and Inverted (I), refer to the orientation of each helianthinin fragment with respect to the truncated 35S CaMV promoter.
- c Developing seeds of transgenic tobacco containing forward constructions in Fig. 1 were assayed for GUS activity at approximately 2 days intervals from 8-24 DPF. Type I, II and  
10 III profiles are defined in Example 3.
- d ND, not determined in this experimental series.
- e In all experiments, GUS assays represent averages from four to ten independently transformed plants for each construction. Standard deviations are included.
- 15 f GUS activity in mature (30 DPF) transgenic tobacco seeds.
- g Transgenic tobacco seedlings were grown axenically on solid medium. Tissues from seedlings (18-20 DPI) were collected and assayed for GUS activity.
- 20 h FL ABA responsive only in developing seeds 12-18 DPF (see text and Table 3). All others, ABA response predicted from GUS expression of dessicated leaves and subsequent demonstration that seedlings of indicated plants respond directly to exogenous ABA. Plus sign indicates induction of GUS activity over basal level. Minus sign indicates no detectable induction of GUS activity.

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EXAMPLE 3Biochemical Detection of GUS Activity:  
Temporally-Regulated Expression

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5 The temporal profile conferred by each forward construction was determined and the results are shown in Table 2. Filial homozygous plants were grown and allowed to flower, and seeds from staged pods were assayed for GUS expression as described in Example 2. Three types of developmental profiles were identified based on the time of initial appearance of GUS activity in developing embryos and the qualitative and quantitative characteristics of the resulting expression patterns; Type I profiles showed correct temporal regulation where accumulation of GUS begins 12 DPF. In plants exhibiting Type II profiles, GUS activity also began accumulating around 12 DPF but peaked around 14 DPF followed by significant declines in levels of GUS activity. Type III plants showed activity occurring before 10 DPF with a peak of activity occurring at approximately 12 DPF. Constructions containing the regions of the HaG3-A URE from nucleotides -2377 to -1527 or -739 to -75 conferred this temporally earlier profile.

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EXAMPLE 4Histochemical Localization of GUS Activity

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5 GUS activity was histochemically localized in seedlings containing HaG3-A-SB/R and HaG3-D-404N. Samples were washed in 50 mM NaPO<sub>4</sub> and incubated for 24 h at 37°C in 100µl reaction buffer [50 mM NaPO<sub>4</sub>, pH 7.0, 2 mM 5-bromo-4-chloro-3-indolyl-glucuronide (X-Gluc), 0.1 mM potassium ferricyanide, and 0.1 mM potassium ferrocyanide]. Samples were mounted on microscope slides with 80% glycerol.

10 HaG3-D-404N (Fig. 7A) and HaG3-A-SB/R (Fig. 7B) seedlings grown on basal media containing 1% sucrose showed slightly different patterns of expression. HaG3-D-N driven GUS expression appeared at low levels in the cotyledons and at significantly higher levels in the distal root region with no detectable activity in the hypocotyl. The HaG3-A-SB/R seedling also showed significant GUS activity in the distal root with no detectable activity in the hypocotyl or cotyledons. GUS activity was histochemically localized at 14 DPI in seedlings containing HaG3-D-404N that were grown in a water-deficient environment on sub-saturated filter paper; GUS activity was primarily in the leaves and roots of these seedlings (Fig. 7C).

15 The GUS expression patterns of seedlings containing HaG3-A-SB/R was determined. The major site of GUS activity in the SB/R seedling was in the developing root tips (Fig. 7B, C). In 6 DPI seedlings containing HaG3-A-SB/R, GUS was expressed throughout the length of the elongating root with particularly high levels in the meristematic region of the root tip (Fig. 7D). Histochemical localization of HaG3-A-SB/R seedlings (14 DPI) showed activity in newly formed lateral roots as well as the continued activity in the meristematic region of the main root (Fig. 7B). Seedlings from 16 DPI continued to show this

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1 pattern of expression (Fig. 7C); root hairs and the distal  
portions of the root had high levels of GUS activity as well.

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EXAMPLE 5ABA-Responsive Expression

In a series of whole plant experiments on transgenic tobacco containing constructions illustrated in Fig. 4, several regions of the UREs of HaG3-A and HaG3-D were identified that responded to changes in the plants water potential (Table 2). Since ABA is a known mediator of water-deficit responses, the effect of ABA on GUS expression driven by these elements was determined. Within HaG3-A, two regions (-1527 to -739 and -739 to -75) were shown to confer ABA-responsive expression in leaves of mature transgenic tobacco and in seedlings. Another ABA-responsive element was identified in the URE of HaG3-D (-739 to -322).

The induction of GUS activity in transgenic tobacco containing HaG3-D-404N (forward orientation) was correlated with water potential during processive desiccation and subsequent recovery from water deficit. Since the full length HaG3-A URE is not expressed under any conditions except during seed development, plants containing this chimeric GUS construction were used as negative controls. Filial, homozygous plants containing each construction were grown in soil. Plants were either watered normally (control) or stressed to varying degrees by watering with 1/3 the amount of the control plant or by not watering at all. Fully stressed plants containing HaG3-D-404N were induced rapidly with a peak of GUS activity at about 36 hours, which correlated with a decrease in water potential (Fig. 8). Subsequent GUS determinations 24 hours later revealed a reproducible decrease in GUS activity even though the plants were under severe water-deficit with water potential of nearly -4 bars. The fully stressed plants were recovered by watering after sampling was completed on day 3. The plants recovered quickly as the water potential returned to non-stressed levels

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1 after watering, and GUS activity continued to decrease over the  
remaining days. GUS activity in 1/3 stressed plants containing  
HaG3-D-N increased more moderately during a 3.5 day interval as  
5 the water potential decreased (Fig. 8). As observed with fully  
stressed plants, GUS activity decreased before water-deficit  
recovery. In no instance did the FL plants express GUS in non-  
embryonic tissues.

To determine if the 404 bp fragment from HaG3-D  
responds directly to ABA, leaf disks of transgenic tobacco  
10 containing HaG3-D-404N were treated with ABA for increasing  
periods of time and were subsequently assayed for GUS  
expression. After a lag-time of approximately 3.5 hours,  
treatment with 10 mM ABA resulted in a rapid increase in GUS  
expression; GUS continued to accumulate through eight hours at  
15 which time the rate of accumulation decreased significantly  
(Fig. 9). There was no detectable GUS activity in leaf disks  
from the same plant maintained under identical conditions  
exclusive of ABA. Likewise, leaf disks from plants containing  
the HaG3-A full length URE showed no activity during the course  
20 of the experiment. Since the chimeric gene including the  
CaMV35S promoter and the  $\beta$ -glucuronidase reporter gene is  
transcriptionally active in leaves (Table 1), transgenic plants  
containing pBI121 served as an important negative control. Leaf  
disks from plants containing pBI121 showed no increase in GUS  
25 activity in response to exogenous ABA throughout the experiment  
(+ABA:  $12.6 \pm 3.3$  pmole 4-MU/ $\mu$ g/min; -ABA:  $13.5 \pm 3.6$  pmole 4-  
MU/ $\mu$ g/min).

A similar series of experiments was carried out with  
transgenic tobacco seedlings containing HaG3-D-404N and HaG3-A-  
30 FL (Fig. 4). Eighteen DPI seedlings were transferred to media  
containing 0-10 mM ABA, and GUS activity was determined one, two  
and three days later (Table 3). Seedlings containing HaG3-D-  
404N were inducible by ABA by day 1 at all ABA concentrations;

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-36-

1 there was no significant induction of HaG3-A-FL in parallel  
experiments. Induction was concentration and time dependent.  
Maximum induction, exceeding 200 fold, occurred at two and three  
days at ABA concentrations of 10 mM (Table 3). Significant  
5 induction of 19 and 70 fold occurred on day three at 0.1 mM and  
1.0 mM ABA, respectively.

The full-length (FL) helianthinin HaG3-A URE (-2377  
to +24) was tested for its inducibility by ABA in developing  
seeds. Seeds containing the full length (FL) regulatory region  
10 driving the expression of GUS (Fig. 4) were staged at 11, 14,  
18 and 24 days post flowering and were tested for their ability  
to respond to ABA. Induction by ABA was shown by the increased  
levels of GUS activity over levels obtained on basal media;  
results are summarized in Table 3. ABA responsiveness varied  
15 with the stage of development. Seeds from 11 DPF did not  
respond to ABA during the course of the experiment whereas more  
mature seeds did respond. Seeds from 14 DPF responded rapidly  
with induction above basal levels beginning as early as 1.5  
hours. There was a monotonic increase in GUS activity with 14  
20 DPF seeds treated with ABA; by three days of treatment, the  
levels of GUS activity were higher than that for 18 and 24 DPF  
seeds treated with or without ABA. Seeds from 18 DPF were  
slower to respond to ABA than those from 14 DPF, but levels of  
GUS activity comparable to 14 DPF (+ABA) seeds were observed in  
25 18 DPF seeds by the fifth day of ABA treatment. Seeds from 24  
DPF are less responsive to ABA through five days of ABA  
treatment. Levels of GUS activity also varied with seeds  
incubated on basal media alone. Seeds from 14 DPF on basal  
media continued to increase in GUS activity an estimated 4 pmol  
30 4-MU/seed/day.

The preceeding results demonstrate a hierarchy  
controlling helianthinin gene expression so that the ABA-  
responsive elements contained within the HaG3 UREs are

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1 functional only within the context of the appropriate  
developmental program, i.e. seed maturation. Taking the ABA-  
responsive elements out of the context of the HaG3-A or HaG3-D  
5 UREs results in the loss of hierarchical control so that these  
elements are free to respond directly to ABA and indirectly to  
desiccation in leaves and seedlings of transgenic tobacco.

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TABLE 3  
ABA Induction In Vitro of HaG3-A-FL in  
in Transgenic Tobacco Seeds"

GUS Activity (pmole 4-MU/μg/min)									
DPF	Normal	11 DPF		14 DPF		18 DPF		24 DPF	
		ABA							
		+	-	+	-	+	-	+	-
11	0	0	0	--	--	--	--	--	--
14	7±0.3	0	0	7.0	7.0	--	--	--	--
16	--	0	0	--	--	--	--	--	--
17	--	--	--	33	15	--	--	--	--
18	15±0.3	--	--	--	--	15	15	--	--
19	--	--	--	57	24	--	--	--	--
21	--	--	--	--	--	24	15	--	--
23	--	--	--	--	--	61	15	--	--
24	16±2.0	--	--	--	--	--	--	16	16
27	--	--	--	--	--	--	--	21	16
29	--	--	--	--	--	--	--	24	16
35	14±2.0	--	--	--	--	--	--	--	--

a Transgenic tobacco seeds containing HaG3-A-FL were collected at indicated days post flowering (DPF) and were incubated on basal media alone or basal media containing 1  $\mu$ M ABA. GUS activity was determined after 0, 3 and 5 days of treatment. In vivo expression of HaG3-A-FL-driven GUS in developing seeds (Normal) is shown for reference.



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EXAMPLE 6Introduction of Herbicide Tolerance into Tobacco

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5 The 0.66 kb BalI-SalI fragment from the parental plasmid pHaG3-A-2.3 (Table 1) was linked at its 5' end to a HindIII site and at its 3' end to an EcoRI site. The resulting cassette was substituted for the double CaMV promoter region in the pRPA-BL-410 construct (described in French Patent Appln. No. 91 02872, filed March 5, 1991) by digesting pRPA-BL-410 with HindIII and EcoRI and subcloning the cassette into that vector. 10 The resulting construct, termed pRPA-ML-803, comprises in the transcriptional frame the following elements: the helianthinin regulatory element, optimized transit peptide (OTP), aroA gene, nos terminator.

15 The plasmid pRPA-ML-803 was transferred into Agrobacterium tumefaciens strain EHA101 (Hood et al. (1986) J. Bacteriol., 168, 1291) by triparental mating and the resulting Agrobacterium was used for leaf disk transformation of tobacco.

20 Regenerated tobacco plants, about twenty centimeters tall, were sprayed in the greenhouse with glyphosate formulated as ROUNDUP at a dose of 0.6Kg of active ingredient/hectare. Untransformed control plants were killed when sprayed with this dose of glyphosate. Transformed plants, which were healthy and viable, showed enhanced tolerance to glyphosate exposure.

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## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

- (i) APPLICANT: Thomas, Terry  
Freyssinet, Georges  
Lebrun, Michel  
Bogue, Molly
- (ii) TITLE OF INVENTION: Chimeric Plant Genes Based on Upstream  
Regulatory Elements of Helianthinin
- (iii) NUMBER OF SEQUENCES: 3
- (iv) CORRESPONDENCE ADDRESS:
  - (A) ADDRESSEE: Scully, Scott, Murphy & Presser
  - (B) STREET: 400 Garden City Plaza
  - (C) CITY: Garden City
  - (D) STATE: New York
  - (E) COUNTRY: USA
  - (F) ZIP: 11530
- (v) COMPUTER READABLE FORM:
  - (A) MEDIUM TYPE: Floppy disk
  - (B) COMPUTER: IBM PC compatible
  - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
  - (D) SOFTWARE: PatentIn Release #1.0, Version #1.25
- (vi) CURRENT APPLICATION DATA:
  - (A) APPLICATION NUMBER:
  - (B) FILING DATE:
  - (C) CLASSIFICATION:
- (viii) ATTORNEY/AGENT INFORMATION:
  - (A) NAME: McNulty, William E.
  - (B) REGISTRATION NUMBER: 22,606
  - (C) REFERENCE/DOCKET NUMBER: 8081
- (ix) TELECOMMUNICATION INFORMATION:
  - (A) TELEPHONE: 516-742-4343
  - (B) TELEFAX: 516-742-4366

## (2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 2401 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: both
  - (D) TOPOLOGY: linear

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GGATCCTCTA CCTATATATA TATATATATA TGAATTTTTT AAAAAAATCC CGTACCCCTC	60
GAAAAAACGG GCCTTATGCG GAAGTCCTCC TCGCACACCT AAAGAGCCGC CCATGCTTTT	120
TAATCAAATA GATGTGCATC ATGTAGTGAT AGTTTTTACT AAAATCCATT AGTTTATAAA	180
TATTTTAAAT GTTTTTTTTT GTTTATATAA AAAAAGAAAA TTAAAAACA AAATGTCCAA	240
AATACTCCTG TATCAACTAT GCAAAAAGAC AAAAAAACC TTTTGGTTAA CAAAGTCTTT	300
AATTTAACTA AGTTTGTCAT TTGAAGGAAA TTCAAACAAA AACGAACGTG GGGGCGCGGG	360
GGTGGGGTGT TTGGTTACAA AAAGTTTTAA TTTTAGATTA AAGTATAAAA ATTGCCCAAA	420
CCTCAGGACA ATTTTACAT TTATAACTCA TTGTCTAAAT ACTAAAATAC ACCAAGTCAA	480
TGGGTGAAAG TTAATATCTT TTTTATTGCA ATTTACACATT ACCTTATTTA CTTTGTAGAA	540
AGACGACATA ACAATTAAGG AGTTATAGTC TGATCGGTTT GCGCTATTTT TCATACTTAA	600
GGTCCAGGTT TGAATCTTTT AAACATTTTT TTTTAACTTG ATCATAACAA TATAACAATT	660
AAGGAGTTAT GATCTGATGG TTTGCGTTAT GTTTTCGTAC TAATTAAGGT CCCGGTTTGA	720
ATCTCTCAA CAATATATTA TTTTTCTTA AAAACGAATG AGACATGCTC ACAATGGGAA	780
TTGAACCGAC ACCTATTGGT TTAAAATTAA AGCTATAACA AACTGAGCTA CACATTTTTA	840
ATTTAAAAAT GTCGACTATC TTAGTTAATC AAATAAATTT ATTTTGATTT GTTTGTGTTA	900
TGTATTTTCT CCTAATTTAA AGTCGATGTG TATTTATATA ATATTAGTAA TATTTTATTA	960
ACATCAATAC ATGCTTCAGG TTTTGTGTTA GTCTTCGTTT TTTATATGGT TTTATCAGTG	1020
GTGTGGTGTA CGATGACGAT TATTTAAATA ATGACGAACT TCTTGGTTGT TACTTATTGA	1080
TGTACGAAGC TGAGATGTAA CGAACCGAAC ACATATAAAT AACATTTTGG ATAAGATTAC	1140
GACTTTATTT ATCGGTTGCC ATGAAATTTA GAAGATTTGG GTTAAGACAC AACCACATAT	1200
AATGTGATGG TAAATAGCAT TTACAACATA TGTTAATCTT TTGTTACAAA TGTTGTTAAC	1260
TAGGCTTGAT ATGTAAAATT TTTAAAGACT ATCAGGTGTT CTTACGGTTT TACATCTAGT	1320
AAGAGATTAA AAAAAAAAAA GCAAGGAAAG TAAGTGTAAG GAGAGTAAAG AGAATGTAGC	1380
CATGATATGG CTGATTGTTT ATCACCATCC CATTTATACT TATCATCTTG ATGATGCATA	1440
TAGACATGAT GTGTGCTACG TACCGAATTT TAACAGCTTC CCGGCGCAAC ACACGTGTAT	1500
AAATACCATA GATTATAAAC CAAATACGCT ACGTATAGGT GGTATATATGA TACCTATGAT	1560
GACTTGACCT TTCGTTACAC TTGAGCTGAA AAAAATAAAA AAATGTGGCT ATAGGCGCAT	1620

GGTCACAGTT TTTTGTGTG GCCATATACA ATTTTGTGACG TAGCGTTAGT TAATCAGATA	1680
AATTTATTTT GATTTGTTTT GTTAATGTAT TTTCTCCTAA TTTCAAGTAG ACGTGTATTT	1740
ATATAATATT AGTAATATTT TATTAACATC AATACATGCT TCATGTTTTG GGTTAGTCTT	1800
CGTTTTTTTAT ATGGTTTTTAT CAGTGGTGTA CGATGACGAT TATTTAAATA ATGACGGACT	1860
TCTTGTTGT TACTTATTGA TGTACGAAGC TGAGATGTAA CGAACCGAAC ACATATAAAT	1920
AACATTTTGG ATAAGATTAC GACTTTATTT ATCGGTTGCC ATGAAATTTG GAAGACTTGG	1980
GTTAAGACAC AACCACATAT AATGTGATGG TAAATAGCAT TTACAACTAA TGTTAATCTT	2040
TTGTTACAAA TGTTGTTAAC TAGGCTTGAT ATGTAAAATT TTAAAGACT ATATGGTGTT	2100
CTTACGGTTT TACATCTAGT AAGAGATTAA AAAAAAAAAA AAAAGCAAGG AAAGTAAGTG	2160
TAAAGAGAGT AAAGAGAATG TAGCCATGAT ATGGCTGATT GTTCATCACC ATCCCATTTA	2220
TACTTATCAT CTTGATGATG CATATAGACA AACACACTAC TTATACAGAT GTAGCATGTC	2280
TCAGCTCCAA ATGGTGATCT TCTCCTGGCA TAACCTCTTA GATGTCACCT CCTCCTTGAT	2340
CTTCTTCCAC TATAAAACCA GCTAGTTCAC AACACCTATT CACCACATCA CATCCCATTC	2400
C	2401

## (2) INFORMATION FOR SEQ ID NO:2:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1732 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: both
- (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

GGATCCTGTA AGAAGTGCCC AAAATGTGAG AAGTGTTATTA TAACACTATA TATAATACTA	60
TATAACACCA TATAAATACC GTATAACACT ATGTAACACC ATATAACACA ATATAACGCT	120
ATGTAACACT ATATAACATT ATATAACAAT ATATAACACT ATACATCTAT CAGAGACATG	180
CTATCAGACA ACCTATAGTG TTATATTTGT TATATAATGT TATATAGTGT TACATAGCGT	240
TATATGGTAT TATATGGTGT TACATATTGT TATACGTGTT TATATGGTGT TATATAGTAT	300
TATATATAGT GTTATAATAC ACTTCTCACA CTTTGGGCAC TTTTACAGG ATCATCTACC	360
TATATATATA TATATATATA TAAAGGATTA GGTTCAAACG TGAACAAATT CCCAAGAGTG	420
AACTGCGTGA ACTGATCTCA GCCCTTGATT TTTATGATCT TGAGATTAAA GTGAGTGCGA	480

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TGATGGTAAT TATTTGGTTA ATTTTTTTTC ATTTAATTAA ATACAAAAG GGTATATGTG	540
TAATTTCAAT CTAAATTGA TTGCATAAAT CTCTCACAAA TCAAGTAATC AATTATCTTC	600
TTAAACTGAT TACATAAATC TCTCACAAAT CAAATCAAGG ATTAGGAAAG ATGTAACCTA	660
ATTCTAATTA CTAAAATAAC TATTTGTTTA AATGCGATGT ACACATGTGT ATTCTGATTT	720
TGCCCTCTTT TTAATGTGAT GTACACATGT GSTATATCGTC TGTTTTTATG AGATCTCAGA	780
ATTTTTTTTTG TATTGAATGT TGATGTACAC CTGTGAATTA CTGTACACAT ATGTACGATG	840
CTGATGCTGA GTACACATGT GTACTGTTCT ATTTATATCC AAGTACACAT GTGTAACCTT	900
GAAATATGAA AGTTACGTGG ATCTTAAAAA TCAAATTTG AATTCTGGTG ATGAAATCTG	960
AAATAAAAAT TAAAATTGAA ATCTGGTGAT TTGTTGTTTG TTTTGATAAT TATCTTATTA	1020
ATAAATAAAC ATAATGTGGA TAATGAATTT AAATTAGGAA AGATGTAACCT TAATTCAATT	1080
ATTAAAATAA TGATTTAAAT CTAATTTTTT ATATAATTAC AATCCTACCC TTAACAACCTA	1140
AAAAGGAAAT CAAGGGTTCA TATCTGTTCA CGCAGTTCAC TCTTGGGAGG TTGTTACGCG	1200
TGGAACCCTA CCCTATATAT ATATATATAT ATATATCAAA TTTTTTTTAAA AAATCCCGTA	1260
CCCCTCGAAA AAACGGGCCT TATGCGGAAG TCCTCCTCGC ACACCTAAAG AGCCGCCCAT	1320
GCTTTTGATC AAATAGTTGT AAATACTAAA ATACACCAAG TCAATGGGTG AAAGTTACTA	1380
TCTTTTTTAT TGCAATTTCA CATTACCTTA TTTACTTTTG AGAAAGACGA CATAACAATT	1440
AAGGAGTTAT AGTCTGATCG TTTGCGCTAT TTTTCATACT TAAGGTCCAG GTTTGAATAT	1500
TTTAAACATT TTTTTTAACT TGATCATAAC AATATAACAA TTAAGGAGTT ATGGTCTGAT	1560
GGTTTGCATT ATGTTTTTCGT ACTAATTAAAG GTCCCGGTTT GAATCTCTCA AACAATATAT	1620
TATTTTTTACC TAAAAACGAA TGAGGCATGC TCACAATGGG AATTGAACCG ACACCTATTG	1680
GTTTAAAATT AAAGCTATAA CAACTGAGC TACACATTTT TAATTTAAAA AT	1732

(2) INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 404 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: both
  - (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GTCGACTATC TTAGTTAATC AAATAAATTT ATTTTGATTT GTTTTGTTAA TGTATTTTCT	60
---	----

CCTAGTTTAA	AGTCGATGTG	TATTTATATA	ATATTAGTAA	TATTTTATTA	ACATCAATAC	120
ATGCTTCAGG	TTTTGTGTTA	GTCTTCGTTT	TTTATATGGT	TTTATCAGCG	GTSTSTSTTA	180
CGATGACGAT	TATTTAAATA	ATGACGGACT	TCTTG GTTGT	TACTTATTGA	TGTACGAAGC	240
TGAGATGTAA	CGAACCGAAC	ACATATAAAT	AACATTTTGG	ATAAGATTAC	GACTTTATTT	300
ATCGGTTGCC	ATGAAATTTG	GAAGACTTGG	GTTAAGACAC	AACCACATAT	AATGTGATGG	360
TAAATAGCAT	TTACAACATA	TGTTAATCTT	TTGTTACAAA	TGTT		404

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1     WHAT IS CLAIMED:

5             1. An isolated nucleic acid from a helianthinin gene comprising at least one regulatory element which directs at least one of seed-specific gene expression, root-specific gene expression, abscisic acid (ABA)-responsive gene expression and temporally-altered gene expression.

10            2. The nucleic acid of Claim 1 wherein said helianthinin gene is selected from an Ha2 gene, an Ha10 gene, an HaG3A gene or an HaG3D gene.

             3. The nucleic acid of Claim 1 wherein said regulatory element is selected from an Ha2 gene, an Ha10 gene, an HaG3A gene or an HaG3D gene.

15            4. The nucleic acid of Claim 1, 2 or 3 wherein the regulatory element which directs seed-specific gene expression is characterized in that expression of a gene under its control is detectable in seeds.

20            5. The nucleic acid of Claim 1, 2 or 3 wherein the regulatory element which directs root-specific gene expression is characterized in that expression of a gene under its control is detectable in plant roots.

25            6. The nucleic acid of Claim 1, 2 or 3 wherein the regulatory element which directs ABA-responsive gene expression is characterized in that expression of a gene under its control is detectable in response to treatment with ABA or conditions which induce ABA biosynthesis.

30            7. The nucleic acid of Claim 1, 2 or 3 wherein the regulatory element which directs temporally-altered gene expression is characterized in that expression of a gene under its control is detectable in plant seeds as early as 4 days post-flowering.

35

1           8. The nucleic acid of Claim 1 or 4 wherein said  
regulatory element directs seed-specific gene expression and  
comprises nucleotides 1 to 2401 or 851 to 2401 of SEQ ID NO:1.

5           9. The nucleic acid of Claim 1 or 5 wherein said  
regulatory element directs root-specific gene expression and  
comprises nucleotides 1 to 1639 or 851 to 1639 of SEQ ID NO:1.

10          10. The nucleic acid of Claim 1 or 6 wherein said  
regulatory element directs ABA-responsive gene expression and  
comprises nucleotides 1 to 2401 of SEQ ID NO:1, nucleotides 851  
to 1639 of SEQ ID NO:1, nucleotides 1639 to 2303 of SEQ ID NO:1  
or nucleotides 1 to 404 of SEQ ID NO:3.

15          11. The nucleic acid of Claim 1 or 7 wherein said  
regulatory element directs temporally-altered gene expression  
and comprises nucleotides 1 to 851 or 1639 to 2303 of SEQ ID  
NO:1.

20          12. The regulatory of any one of Claims 1-11 wherein  
said regulatory element is operably linked to the coding  
sequence of a heterologous gene to effect said expression of a  
gene product from said coding sequence and to provide a chimeric  
plant gene.

25          13. The chimeric plant gene of Claim 12 comprising  
a sufficient part of a promoter capable of functioning in plants  
and operably linked to said coding sequence and said regulatory  
element to effect expression of said heterologous gene.

30          14. The chimeric plant gene of Claim 12 wherein said  
promoter is a plant virus promoter or the cauliflower mosaic  
virus (CaMV) 35S promoter.

35          15. The chimeric plant gene of Claim 14 wherein said  
promoter is the CaMV 35S promoter comprising CAAT and TATA  
sequences.

40          16. The chimeric plant gene of Claim 12 wherein said  
heterologous gene is a gene encoding a lipid metabolism enzyme,  
a desaturase, a herbicide resistance gene, a glyphosate



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1 resistance gene or a gene encoding 5' enolpyruvylshikimic acid-  
3-phosphate synthase, acetolactase synthase, or acetohydroxy  
acid synthase.

5 17. The chimeric gene of Claim 16 wherein said  
glyphosate resistance gene is aroA.

18. The chimeric gene of Claim 17 which comprises the  
chimeric plant gene of pRPA-ML-803.

19. A plant transformation vector which comprises the  
chimeric plant gene of any one of Claims 12-18.

10 20. A plant cell comprising the transformation vector  
of Claim 19.

21. A plant, or a progeny of said plant, which has  
been regenerated from the plant cell of Claim 20.

15 22. The plant of Claim 21 wherein said plant is a  
cotton, tobacco, oil seed rape, maize or soybean plant.

23. The plant cell of Claim 20 wherein said plant  
cell is a cotton, tobacco, oil seed rape, maize or soybean plant  
cell.

20 24. A method for producing a plant with improved seed  
lipid quality which comprises:

- a) transforming a plant cell with the transformation  
vector of Claim 19; and
- b) regenerating said plant with improved seed lipid  
quality from said transformed plant cell.

25 25. A method for producing a plant which exhibits  
resistance to a herbicide which comprises:

- a) transforming a plant cell with the transformation  
vector of Claim 19; and
- b) regenerating said plant from said transformed  
plant cell.

30 26. The use of regulatory element of any one of  
Claims 1-12 for producing transgenic plants.

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1           27. The use of the nucleic acid of any one of Claims  
1-11 for producing transgenic plants.

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-2377						GGATCCT
-2340	TTTTAAAAAA		TCCCGTACCC		CTCGAAAAAA	
-2280	CCTAAAGAGC		CGCCCATGCT		TTTTAATCAA	
-2220	ACTAAATCC		ATTAGTTTAT		AAATATTTTA	
-2160	AAATTAAAAA		ACAAAATGTC		CAAAATACTC	
-2100	CCCTTTTGGT		TAACTAAAGTC		TTTAAATTTAA	
-2040	AAAAACGAAC		GTGGGGGCGC		GGGGGTGGGG	
-1980	TTAAAGTATA		AAAATTGCCC		AAACCTCAGG	
-1920	AATACTAAAA		TACACCAAGT		CAATGGGTGA	
-1860	ATTACCTTAT		TTACTTTTGA		GAAAGACGAC	
-1800	TTTGCGCTAT		TTTTCATACT		TAAGGTCCAG	
-1740	TTGATCATAA		CAATATAACA		ATTAAGGAGT	
-1680	TACTAATTAA		GGTCCCGGTT		TGAATCTCTC	
-1620	ATGAGACATG		CTCACAATGG		GAATTGAACC	
-1560	ACAAACTGAG		CTACACATTT		TTAATTTAAA	
-1500	TTTATTTTGA		TTTGTTTTGT		TAAATGATTT	
-1440	ATAATATTAG		TAAATATTTA		TTAACATCAA	
-1380	TTTTTTTATAT		GGTTTTATCA		GTGGTGTGGT	
-1320	ACTTCTTGGT		TGTTACTTAT		TGATGTACGA	
-1260	AATAACATTT		TGGATAAGAT		TACGACTTTA	
-1200	TGGGTAAAGA		CACAACCACA		TATAATGTGA	
-1140	CTTTTGTTAC		AAATGTTGTT		AACTAGGCTT	
-1080	GTTCTTACGG		TTTTACATCT		AGTAAGAGAT	
-1020	AAAGAGAGTA		AAGAGAATGT		AGCCATGATA	
-960	ACTTATCATC		TTGATGATGC		ATATAGACAT	
-900	TTCCCGGCGC		AACACACGTG		TATAAATACC	
-840	GGTGGTTATA		TGATACCTAT		GATGACTTGA	
-780	AAAAAATGTG		GCTATAGGCG		CATGGTCACA	
-720	ACGTAGCGTT		AGTTAATCAG		ATAAATTTAT	
-660	TAATTTCAAG		TAGACGTGTA		TTTATATAAT	
-600	GCTTCATGTT		TTGGGTTAGT		CTTCGTTTTT	
-540	GATTATTTAA		ATAATGACGG		ACTTCTTGGT	
-480	TAACGAACCG		AACACATATA		AATAACATTT	
-420	GCCATGAAAT		TTGGAAGACT		TGGGTAAAGA	
-360	CATTTACAAC		TAATGTAAAT		CTTTTGTTAC	
-300	ATTTTTAAAG		ACTATATGGT		GTTCCTACGG	
-240	AAAAAAAGCA		AGGAAAGTAA		GTGTAAGAG	
-180	ATTGTTTCATC		ACCATCCCAT		TTATACTTAT	
-120	TACTTATACA		GATGTAGCAT		GTCTCAGCTC	
-60	TTAGATGTCA		CTTCCTCCTT		GATCTTCTTC	
1	ATTCACCACA		TCACATCCCA		TTCC	
			10		20	
						30

FIG. 1

SUBSTITUTE SHEET

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-30		-20		-10		
CTACCTATAT		ATATATATAT		ATATGAATTT		-2341
CGGGCCTTAT		GCGGAAGTCC		TCCTCGCACA		-2281
ATAGATGTGC		ATCATGTAGT		GATAGTTTTT		-2221
AATGTTTTTT		TTTGTTTATA		TAAAAAAGA		-2161
CTGTATCAAC		TATGCAAAA		GACAAAAAAA		-2101
CTAAGTTTGT		CATTTGAAGG		AAATTCAAAC		-2041
TGTTTGGTTA		CAAAAAGTTT		TAATTTTAGA		-1981
ACAATTTTTA		CATTTATAAC		TCATTGTCTA		-1921
AAGTTACTAT		CTTTTTTATT		GCAATTTTAC		-1861
ATAACAATTA		AGGAGTTATA		GTCTGATCGG		-1801
GTTTGAATCT		TTTAAACATT		TTTTTTTAAAC		-1741
TATGATCTGA		TGGTTTGCGT		TATGTTTTTCG		-1681
AAACAATATA		TTATTTTTTC		TTAAAAACGA		-1621
GACACCTATT		GGTTTAAAA		TAAAGCTATA		-1561
AATGTGCACT		ATCTTAGTTA		ATCAAAATAA		-1501
TCTCCTAATT		TAAAGTCGAT		GTGTATTTAT		-1441
TACATGCTTC		AGGTTTTGTG		TTAGTCTTCG		-1381
GTACGATGAC		GATTATTTAA		ATAATGACGA		-1321
AGCTGAGATG		TAACGAACCG		AACACATATA		-1261
TTTATCGGTT		GCCAIGAAAT		TTAGAAGATT		-1201
TGGTAAATAG		CATTTACAAC		TAATGTTAAT		-1141
GATATGTAAA		ATTTTAAAG		ACTATCAGGT		-1081
TAAAAAANA		AAAGCAAGGA		AAGTAAGTGT		-1021
TGGCTGATTG		TTCATCACCA		TCCCATTTAT		-961
GATGTGTGCT		ACGTACCGAA		TTTTAACAGC		-901
ATAGATTATA		AACCAAAATC		GCTACGTATA		-841
CCTTTCGTTA		CACTTGAGCT		GAAAAAATA		-781
GTTTTTTTGT		GTGGCCATAT		ACAATTTTGT		-721
TTTGATTTGT		TTTGTTAATG		TATTTTCTCC		-661
ATTAGTAATA		TTTTATTAAAC		ATCAATACAT		-601
TATATGGTTT		TATCAGTGGT		GTACGATGAC		-541
TGTTACTTAT		TGATGTACGA		AGCTGAGATG		-481
TGGATAAGAT		TACGACTTTA		TTTATCGGTT		-421
CACAACCACA		TATAATGTGA		TGGTAAATAG		-361
AAATGTTGTT		AAC TAGGCTT		GATATGTAAA		-301
TTTTACATCT		AGTAAGAGAT		TAAAAAANA		-241
AGTAAAGAGA		ATGTAGCCAT		GATATGGCTG		-181
CATCTTGATG		ATGCATATAG		ACAAACACAC		-121
CAAATGGTGA		TCTTCTCCTG		GCATAACCTC		-61
CACTATAAAA		CCAGCTAGTT		CACAACACCT		-1

24

	40		50		60
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FIG. 1 CONT.'

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	-60		-50		-40	
-2457	GGATCCT		GTAAGAAGTG		CCCCAAATGT	
-2400	CTATATAACA		CCATATAAAT		ACCGTATAAC	
-2340	GCTATGTAAC		ACTATATAAC		ATTATATAAC	
-2280	ATGCTATCAG		ACAACCTATA		GTGTTATATT	
-2220	CGTTATATGG		TATTATATGG		TGTTACATAT	
-2160	TATTATATAT		AGTGTTATAA		TACACTTCTC	
-2100	ACCTATATAT		ATATATATAT		ATATAAAGGA	
-2040	GTGAACTGCG		TGAACTGATC		TCAGCCCTTG	
-1980	GCATGATGGT		AATTATTTGG		TTAATTTTTT	
-1920	GTGTAATTTT		AATCTTAAAT		TGATTGCATA	
-1860	TTCTTAAACT		GATTACATAA		ATCTCTCACA	
-1800	TTAATTCTAA		TTACTAAAAT		AACTATTTGT	
-1740	TTTTGCCCTC		TTTTTAATGT		GATGTACACA	
-1680	AGAAATTTTT		TTGTATTGAA		TGTTGATGTA	
-1620	ATGCTGATGC		TGAGTACACA		TGTGTACTGT	
-1560	CTTGAAATAT		GAAAGTTACG		TGGATCTTAA	
-1500	CTGAAATAAA		AATTAALATT		GAAATCTGGT	
-1440	TTAATAAATA		AACATAATGT		GGATAATGAA	
-1380	ATTATTAAAA		TAATGATTTA		AATCTAATTT	
-1320	CTAAAAAGGA		AATCAAGGGT		TCATATCTGT	
-1260	CGCTGGAACC		CTACCCTATA		TATATATATA	
-1200	GTACCCCTCG		AAAAAACGGG		CCTTATGCGG	
-1140	CATGCTTTTG		ATCAAAATAGT		TGTAAATACT	
-1080	CTATCTTTTT		TATTGCAATT		TCACATTACC	
-1020	ATTAAGGAGT		TATAGTCTGA		TCGTTTGC GC	
-960	TATTTTAAAC		ATTTTTTTTA		ACTTGATCAT	
-900	GATGGTTTGC		GTTATGTTTT		CGTACTAATT	
-840	TATTATTTTT		ACCTAAAAAC		GAATGAGGCA	
-780	TTGGTTTAAA		ATTAAGCTA		TAACAAACTG	

FIG. 2

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-30      |   -20      |   -10      |  
GAGAAAGTGTA TTATAACACT ATATATAATA -2401  
ACTATGTAAAC ACCATATAAC ACAATATAAC -2341  
AATATATAAC ACTATACATC ATCAGAGAC -2281  
TGTTATATAA TGTTATATAG TGTTACATAG -2221  
TGTTATACGT GTTTATATGG TGTTATATAG -2161  
ACACTTTGGG CACTTTTAC AGGATCATCT -2101  
TTAGGTTCAA ACGTGAACAA ATTCCCAAGA -2041  
ATTTTTATGA TCTTGAGATT AAAGTGAGTG -1981  
TTCATTTAAT TAAATACAAA AAGGGTATAT -1921  
AATCTCTCAC AAATCAAGTA ATCAATTATC -1861  
AATCAAAATCA AGGATTAGGA AAGATGTAAC -1801  
TTAAATGCGA TGTACACATG TGTATTCTGA -1741  
TGTGTATATC GTCTGTTTTT ATGAGATCTC -1681  
CACCTGTGAA TTAGTGTACA CATATGTACG -1621  
TCTATTTATA TCCAAGTACA CATGTGTAAC -1561  
AAATCAAAAT TTGAATTCTG GTGATGAAAT -1501  
GATTTGTTGT TTGTTTGTAT AATTATCTTA -1441  
TTTAAATTAG GAAAGATGTA ACTTAATTCA -1381  
TTTATATAAT TACAATCCTA CCCTTAACAA -1321  
TCACGCAGTT CACTCTTGGG AGGTTGTTCA -1261  
TATATATATC AAATTTTTTT AAAAAATCCC -1201  
AAGTCCTCCT CGCACACCTA AAGAGCCGCC -1141  
AAAAACACC AAGTCAATGG GTGAAAGTTA -1081  
TTATTTACTT TTGAGAAAGA CGACATAACA -1021  
TATTTTTCAT ACTTAAGGTC CAGGTTTGAA -961  
AACAAATATA CAATTAAGGA GTTATGGTCT -901  
AAGGTCCCGG TTTGAATCTC TCAAACAATA -841  
TGCTCACAAT GGGAAATTGAA CCGACACCTA -781  
AGCTACACAT TTTTAATTTA AAAAT
```

FIG. 2 CONT.'

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-720 CTATCTTAGT TAATCAAAATA AATTTATTTT  
-660 TTTAAAGTCG ATGTGTATTT ATATAATATT  
-600 TCAGGTTTTG TGTTAGTCTT CGTTTTTTAT  
-540 ACGATTATTT AAATAATGAC GGACTTCTTG  
-480 TGTAAACGAAC CGAACACATA TAAATAACAT  
-420 TTGCCATGAA ATTTGGAAGA CTTGGGTTAA  
-360 AGCATTTACA ACTAATGTTA ATCTTTTGTT

FIG. 3

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		GTCGA	-721
GATTTGTTTT	GTTAATGTAT	TTTCTCCTAG	-661
AGTAATATTT	TATTAACATC	AATACATGCT	-601
ATGGTTTTAT	CAGCGGTGTG	GTGTACGATG	-541
GTTGTTACTT	ATTGATGTAC	GAAGCTGAGA	-481
TTTGGATAAG	ATTACGACTT	TATTTATCGG	-421
GACACAACCA	CATATAATGT	GATGGTAAAT	-361
ACAAATGTT			

FIG. 3 CONT.'

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FIG. 4

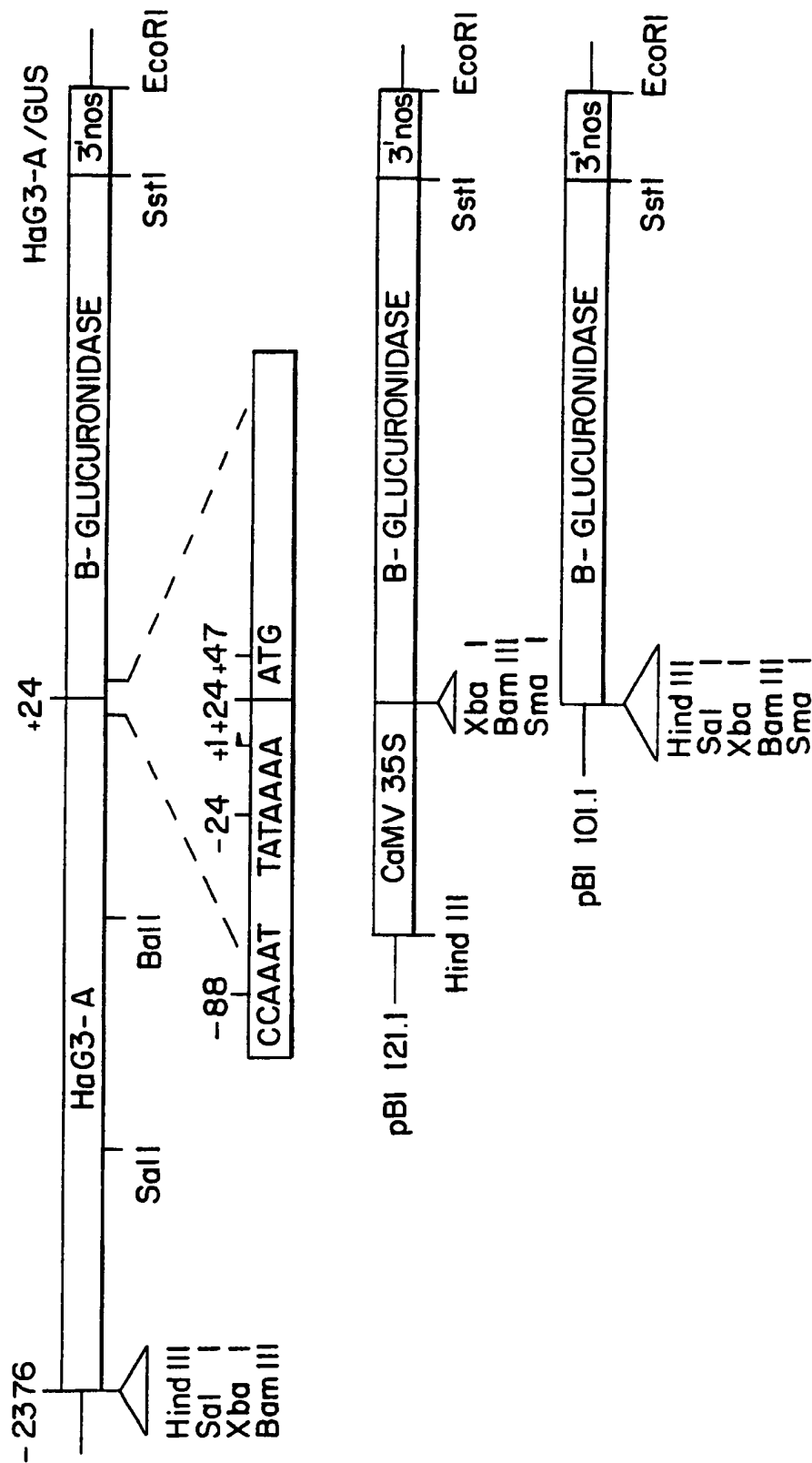


FIG. 5

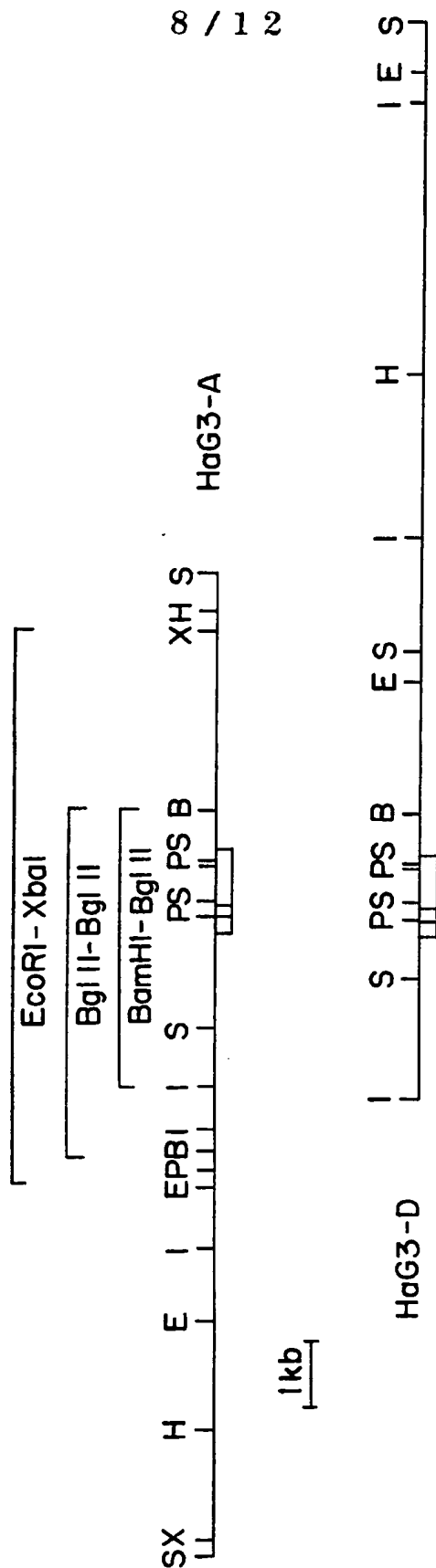
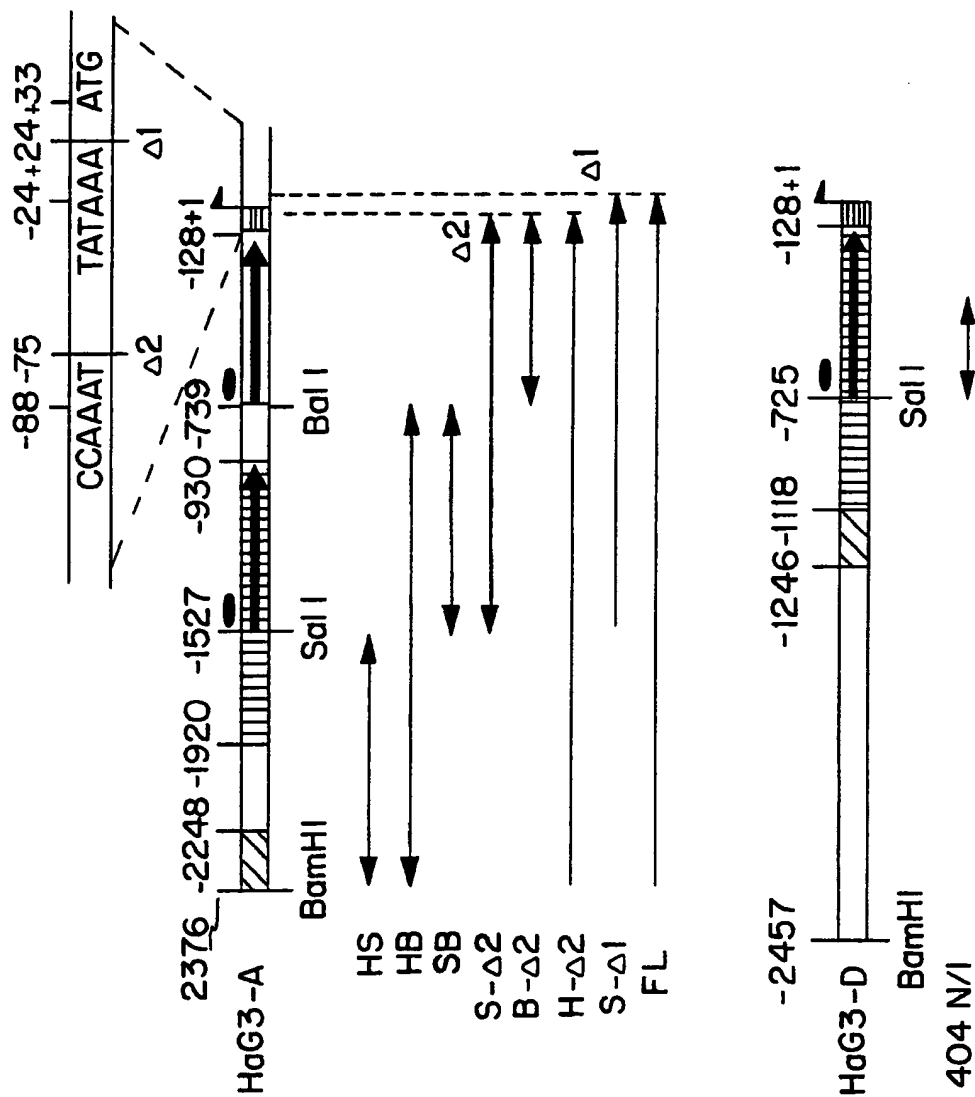


FIG. 6



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FIG. 7A

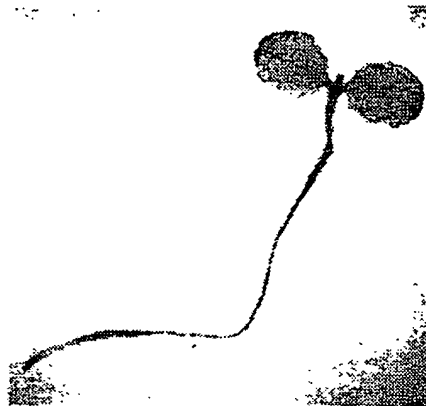


FIG. 7B

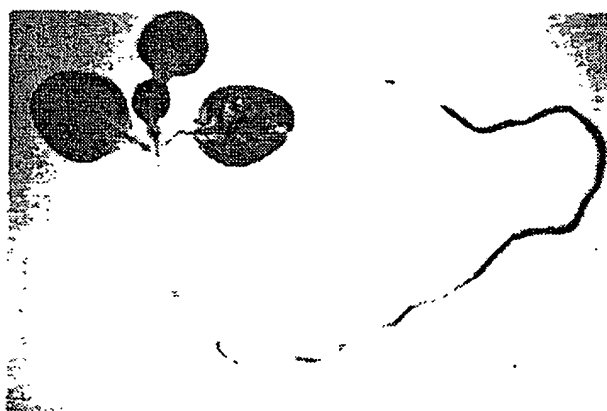


FIG. 7C



FIG. 7D

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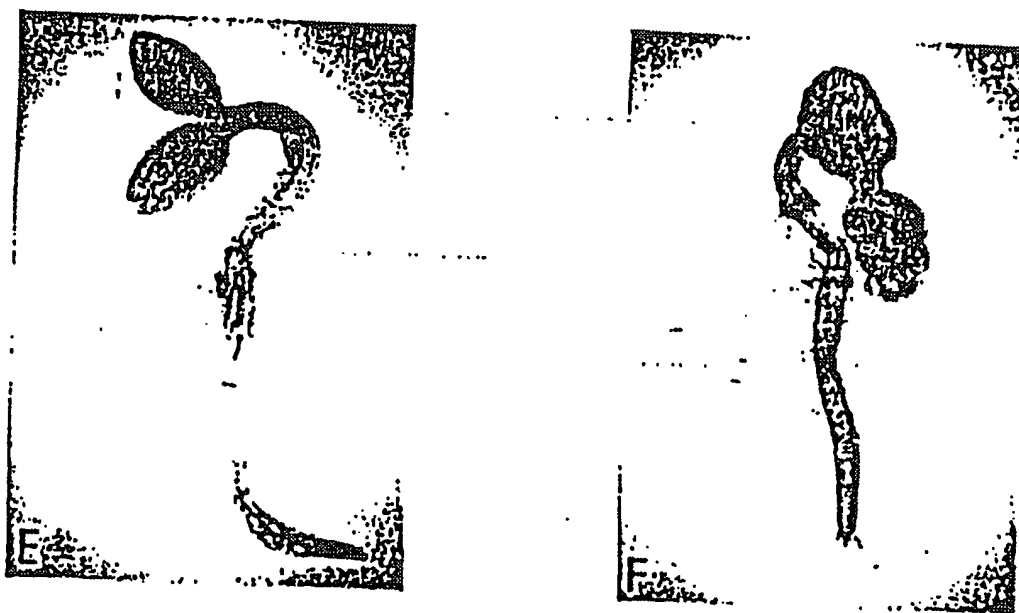


Figure 7

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FIG. 8A

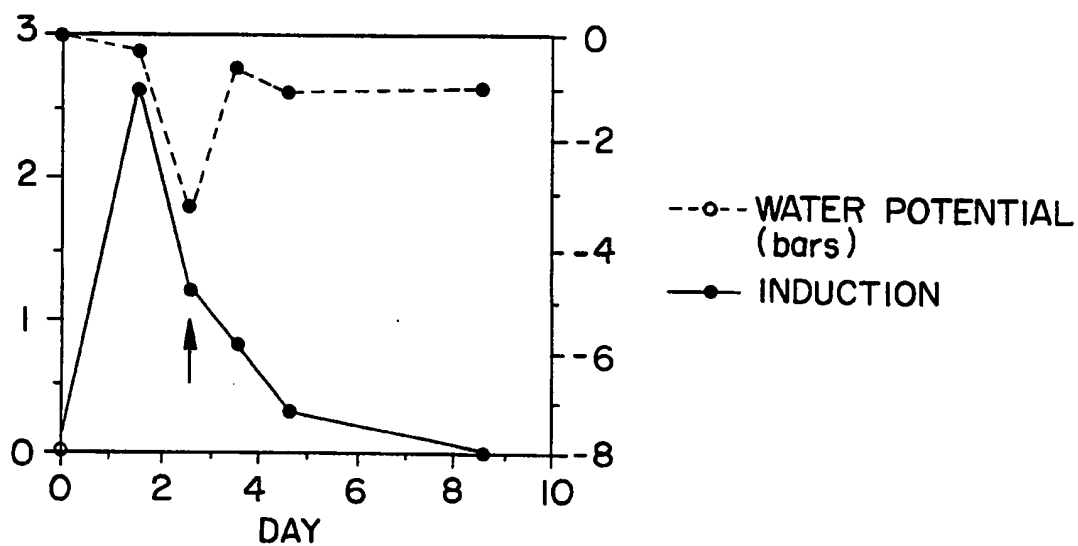
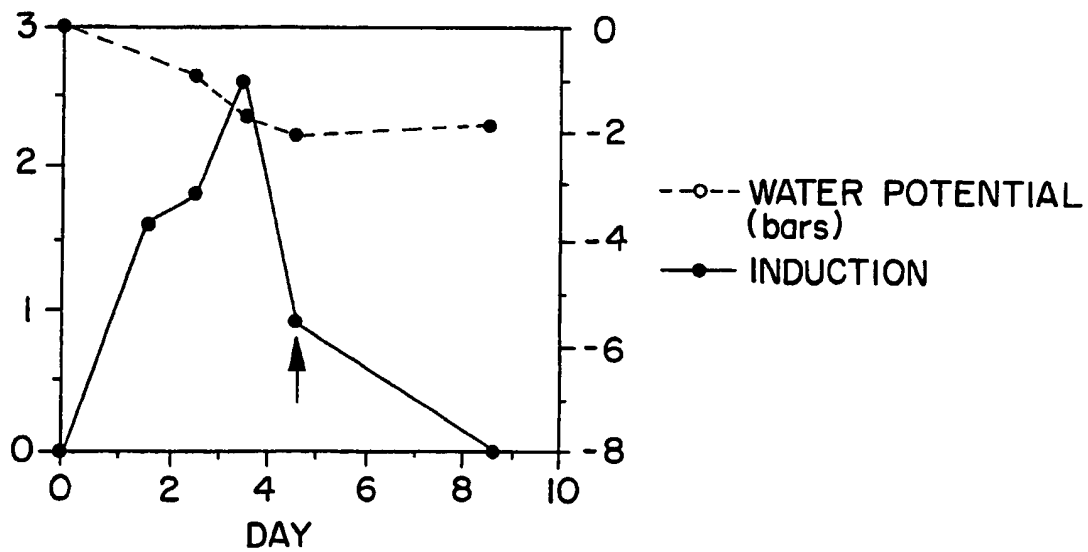
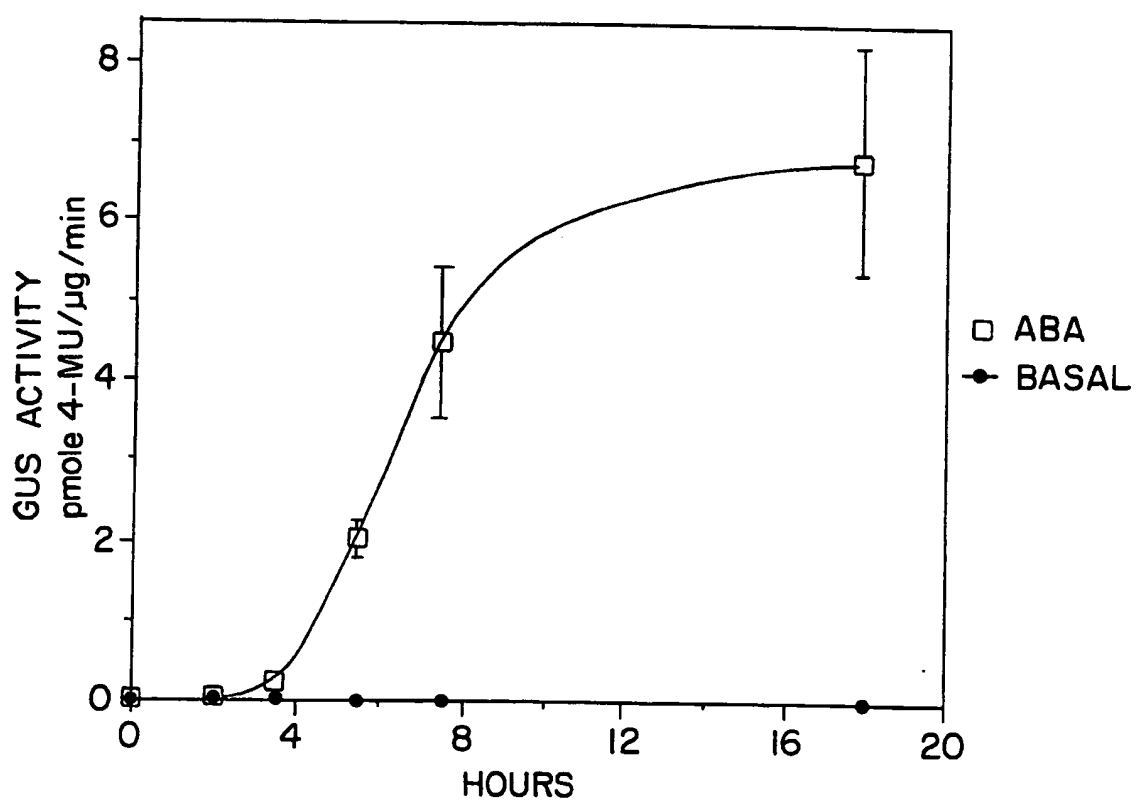


FIG. 8B



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FIG. 9



# INTERNATIONAL SEARCH REPORT

International Application No. PCT/US92/02822

<b>I. CLASSIFICATION OF SUBJECT MATTER</b> (if several classification symbols apply, indicate all) <sup>2</sup> According to International Patent Classification (IPC) or to both National Classification and IPC IPC (5): C12N 05/10, 15/63, 15/82; C07H 15/12; A01H 5/00 US CL : 435/172.3, 240.4, 320.1; 536/27; 800/205		
<b>II. FIELDS SEARCHED</b>		
Minimum Documentation Searched <sup>4</sup>		
Classification System	Classification Symbols	
U.S.	435/172.3, 240.4, 320.1; 536/27; 800/205, DIG. 69; 935/30, 35, 64, 67	
Documentation Searched other than Minimum Documentation to the extent that such Documents are included in the Fields Searched <sup>5</sup>		
APS, DIALOG search terms: helianthinin		
<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT</b> <sup>14</sup>		
Category <sup>15</sup>	Citation of Document <sup>16</sup> with indication, where appropriate, of the relevant passages <sup>17</sup>	Relevant to Claim No. <sup>18</sup>
X/Y	The Plant Cell, Volume 1, issued September 1989, Jordano et al, "A sunflower helianthinin gene upstream sequence ensemble contains an enhancer and sites of nuclear protein interaction", pages 855-866, see entire document.	1-4, 8, 12-15, 19-23/16, 24
X/Y	Gene, Volume 74, issued 1988, Vonder Haar et al, "Organization of the sunflower 11S storage protein gene family", pages 433-443, see entire document.	1-4, 8 / 12-16, 19-24
Y	Trends in Biotechnology, Volume 5, issued February 1987, Knauf, "The application of genetic engineering to oilseed crops", pages 40-47, see entire document.	12-13, 16, 19-24
Y	EP. A, 0,255,377 (Kridl et al) 03 February 1988, see entire document.	12-13, 16, 19-24
Y	Trends in Genetics, Volume 4, No. 1, issued January 1988, Willmitzer, "The use of transgenic plants to study plant gene expression", pages 13-18, see entire document.	14-15
<b>Special categories of cited documents:</b> <sup>19</sup>		
"A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "Z" document member of the same patent family		
<b>IV. CERTIFICATION</b>		
Date of the Actual Completion of the International Search <sup>2</sup> 23 June 1992		Date of Mailing of this International Search Report <sup>2</sup> 07 JUL 1992
International Searching Authority <sup>1</sup> ISA/US		Signature of Authorized Official <sup>3</sup> P. R. RHODES



FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET		
Y	The Journal of Biological Chemistry, Volume 265, No. 33, issued 25 November 1990, Stukey et al, "The OLE1 gene of <i>Saccharomyces cerevisiae</i> encodes the $\Delta 9$ fatty acid desaturase and can be functionally replaced by the rat stearoyl-CoA desaturase gene", pages 20144-20149, see entire document.	16
Y	The Journal of Biological Chemistry, Volume 257, No. 20, issued 25 October 1982, McKeon et al, "Purification and characterization of the stearoyl-acyl carrier protein desaturase and the acyl-acyl carrier protein thioesterase from maturing seeds of safflower", pages 12141-12147, see entire document.	16
<b>V. <input type="checkbox"/> OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE<sup>1</sup></b>		
<p>1. <input type="checkbox"/> Claim numbers , because they relate to subject matter (1) not required to be searched by this Authority, namely:</p> <p>2. <input type="checkbox"/> Claim numbers , because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out (1), specifically:</p> <p>3. <input type="checkbox"/> Claim numbers , because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).</p>		
<b>VI. <input checked="" type="checkbox"/> OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING<sup>2</sup></b>		
<p>This International Searching Authority found multiple inventions in this international application as follows: SEE ATTACHMENT</p> <p>1. <input type="checkbox"/> As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.</p> <p>2. <input checked="" type="checkbox"/> As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims: 1-4, 8, 12-16, AND 19-24</p> <p>3. <input type="checkbox"/> No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:</p> <p>4. <input type="checkbox"/> As all searchable claims could be searched without effort justifying an additional fee, the International Search Authority did not invite payment of any additional fee.</p> <p>Remark on protest</p> <p><input type="checkbox"/> The additional search fees were accompanied by applicant's protest.</p> <p><input checked="" type="checkbox"/> No protest accompanied the payment of additional search fees.</p>		

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category*	Citation of Document, <sup>16</sup> with indication, where appropriate, of the relevant passages <sup>17</sup>	Relevant to Claim No. <sup>18</sup>
Y	US, A. 4,394,443 (Weissman et al) 19 July 1983, see entire document.	16

The claims present a plurality of mutually exclusive independent inventions as follows:

I. Claims 1-3, 12-16, and 19-24, drawn to a DNA product and the vector, plant cell, and plant containing same and a first method of use, classified in Classes 435, 536, and 800; Subclasses 172.3, 240.4, and 320.1, 27, and 205, respectively, for example.

Note that the following are independent and distinct species pertinent to the invention of Group I where a) is the first species of the first component and e) is the first species of the second component and both of which will be searched with claims 1-3, 12-16, and 19-24 in the event that no other fees are paid. Note that a search of any other additional species within Group I requires payment of additional fees.

The first component:

- a) seed-specific regulatory element (claims 4 and 8);
- b) root-specific regulatory element (claims 5 and 9);
- c) ABA-responsive regulatory element (claims 6 and 10);
- d) temporally-altered regulatory element (claims 7 and 11);

The second component:

- e) the heterologous gene encodes a lipid metabolism enzyme;
- f) the heterologous gene encodes a desaturase;
- g) the heterologous gene encodes a herbicide resistance gene;
- h) the heterologous gene encodes *aroA* for glyphosate resistance (claims 17-18);
- i) the heterologous gene encodes EPSPS;
- j) the heterologous gene encodes acetolactase synthase;
- k) the heterologous gene encodes acetohydroxy acid synthase.

II. Claim 25, drawn to a second process of use, classified in Class 435, Subclass 172.3, for example.

III. Claim 26, drawn to a third process of use, classified in Class 435, Subclass 172.3, for example.

IV. Claim 27, drawn to a fourth process of use, classified in Class 435, Subclass 172.3, for example.

The inventions are distinct, each from the other because of the following reasons:

The claims of each of Groups I-IV for a method of use are distinct and independent as the search for each group is not coextensive for the different properties of the diverse products produced by these methods require different considerations for regulating expression and vector construction. Furthermore, the third method of use requires steps to identify regulatory subset sequences as well as a search of separate subject matter since the third and fourth methods of use are unspecified and since there are numerous methods of making transgenic plants and since considerations for construction and operability differ with regulatory element sequences as opposed to whole fragments containing various regulatory elements including silencers, for example. A search for any one group would not result in a complete and thorough search for any one other group.

PCT/ISA/210

PCT/US92/02822

Because these inventions are distinct for the reasons given above and have acquired a separate status in the art as shown by their recognized divergent subject matter and separate search requirements, lack of unity as indicated is proper.